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# **PREVENTION OF PLASMIN-INDUCED HYDROLYSIS OF CASEINS**

A thesis presented in partial fulfilment of the requirements of the degree of Doctor of Philosophy in Food Technology at Massey University, Palmerston North, New Zealand

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# Abstract

Bovine plasmin is a proteolytic enzyme that is naturally present in milk. Plasmin can have a detrimental impact on product quality including proteolysis, age-gelation and bitterness. The activity of plasmin is difficult to control as its precursor, plasminogen, and its activators can survive severe heat treatments such as ultra-high-temperature processing.

The aim of this work was to understand and control the plasmin-induced hydrolysis of caseins in milk systems. A sequential approach was used. In the first stage, the effect of substrate modification on plasmin-induced hydrolysis in a pure  $\beta$ -casein model system was studied; this allowed us to propose a control mechanism to limit the availability of the substrate by protein modification. In the second stage, different protein modifications were applied to a real milk system. In the analysis of this system, the casein micelle structure, whey protein denaturation and whey protein association with the casein micelle were considered. The final stage investigated plasmin-induced dissociation of casein micelles in real milk systems to understand the effect of plasmin activity on gelation and sedimentation in heat-treated milks.

Modification of lysine residues on the protein decreased plasmin-induced hydrolysis. Lactosylation had a greater effect than succinylation and transglutamination at the same level of lysine modification. A mechanism for this phenomenon was proposed. Lactosylation involves the attachment of lactose and, in advanced stages, cross-linking, thus modifying lysine and making it unrecognisable to plasmin; in addition, the cross-linking may affect the release of plasmin-generated peptides. Transglutamination also modifies lysine by cross-linking and has a similar effect to lactosylation, but to a lesser extent. In contrast, succinylation modifies the charge associated with lysine, making it unrecognisable to plasmin. Collectively, this knowledge can be used to make protein resistant to plasmin activity.

The combined effect of micellar structure and protein modification on plasmin activity was also studied. Calcium chelation and dissociation of the casein micelle increased plasmin activity because of reduced steric hindrance, which made the protein more readily available to plasmin. In contrast, succinylation decreased plasmin activity, which could be attributed to the formation of succinyl-lysine rendering  $\beta$ -casein unrecognisable to the substrate-binding pocket of plasmin, resulting in a decrease in hydrolysis with an increase in modification. These results indicated the importance of the casein micelle structure as a tool for controlling the activity of plasmin on milk proteins in food systems.

The effect of high heat treatment on plasmin-induced hydrolysis was also investigated. A high-heat-treated skim milk (120°C/15 min) was found to have greater resistance to plasmin activity than non-heated skim milk. Both whey protein association with the casein micelles and lactosylation decreased the availability of protein to plasmin. Whey-protein-free milk was the most plasmin resistant, followed by skim milk and lactose-free milk. Collectively, these results suggest that lactosylation plays a more significant role than whey protein association with the casein micelles in making protein resistant to plasmin activity.

The plasmin-induced dissociation of the casein micelle was explored by identifying peptide release from the micelle. Upon plasmin-induced hydrolysis of the casein micelle, hydrophilic peptides, i.e. proteose peptones, were the first to dissociate from the casein micelle, followed by hydrophobic peptides, which had dissociation patterns that were identical to those of  $\kappa$ -casein. This suggests that the release of  $\kappa$ -casein from the micelle is too slow to cause gelation. Extensive plasmin-induced hydrolysis of the casein micelle leads to sedimentation in heat-treated milk because of the formation of  $\beta$ -lactoglobulin- $\kappa$ -casein complexes and their aggregation with hydrolysed hydrophobic peptides.

Overall, the results of the present study showed that casein modification can be useful in controlling plasmin activity and has developed our understanding of the plasmin-induced dissociation of casein micelles. Further research work is needed to understand the mechanism of plasmin's selective hydrolysis pattern and the

structural aspects of the substrate-binding pocket of plasmin. Studies on casein micelle dissociation separately and in conjunction with physicochemical changes during storage could be useful in further understanding the phenomenon of age gelation.



To my wife Namrata and son Sharav  
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## Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: \_\_\_\_\_

Date: \_\_\_\_\_



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## List of Conference Paper/Poster Presentations and Publications

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Bhatt, H., Cucheval, A., Coker, C., Patel, H., Carr, A., & Bennett, R. (2013e). *The mechanism of resistance to plasmin activity through protein succinylation: a model study using  $\beta$ -casein*. Poster #T217 presented at the ADSA–ASAS Joint Annual Meeting, 08–12 July 2013, Indianapolis, IN, USA.

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Bhatt, H., Cucheval, A., Coker, C., Patel, H., Carr, A., & Bennett, R. (2014b). Effect of lactosylation on plasmin-induced hydrolysis of  $\beta$ -casein. *International Dairy Journal*, 38(2), 213–218. doi: 10.1016/j.idairyj.2014.01.017.

Bhatt, H., Cucheval, A., Coker, C., Patel, H., Carr, A., & Bennett, R. (2014c). *Effect of micellar structure of casein and their modification on plasmin-induced hydrolysis*. Paper and poster presented at the IFT Conference 2014, New Orleans, LA, USA. – **Won second prize for the best oral presentation.**

Bhatt, H., Cucheval, A., Coker, C., Patel, H., Carr, A., & Bennett, R. (2014d). *Elucidating the role of plasmin in sedimentation and age gelation in a heat-treated milk system*. Poster presented at the IFT Conference 2014, New Orleans, LA, USA.

Bhatt, H., Cucheval, A., Coker, C., Patel, H., Carr, A., & Bennett, R. (2014e). *Interactions of whey protein with casein play a major role in preventing plasmin-induced hydrolysis in heated milk system*. Poster presented at the 7<sup>th</sup> International Whey Conference, 2014, Rotterdam, Netherlands.

Bhatt, H., Cucheval, A., Coker, C., Patel, H., Carr, A., & Bennett, R. (2014f). *Plasmin-induced dissociation of the casein micelle: effect of casein micelle*

*modification on plasmin-induced hydrolysis of skim milk.* Paper presented at the ADSA–ASAS Joint Annual Meeting, 2014, Kansas City, MO, USA.



# Chapter 1: Introduction

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## 1.1 PROBLEM DEFINITION

Fonterra is one of the leading manufacturers and exporters of casein-based ingredients such as milk protein concentrates (MPCs), caseins and caseinates. These casein-based ingredients have increasingly been used in various applications, including neutral-pH beverages, long-shelf-life ultra-high-temperature (UHT)-treated milks, yoghurts, cheeses and many other products. One of the inherent problems is that, during the manufacture and storage of casein-based ingredients such as MPC, the protease system (the plasmin system), which is naturally present in milk, is also concentrated. Therefore, the plasmin activities in such ingredients can be very high.

The protease system occurs in milk, is very complex and includes plasminogen, plasmin, plasminogen activators (PAs), plasminogen activator inhibitors (PAIs) and plasmin inhibitors (PIs). The components of this system have different heat labilities and, when considered as a whole, the system has a moderate resistance to heat inactivation. In products such as UHT milk reconstituted from low-heat skim milk powder or MPC, it is possible that the PAs are free to facilitate the conversion of plasminogen to plasmin during storage because of their survival during heat treatment. The presence of plasmin activity is dependent on favourable conditions for the activation of plasminogen to plasmin in the product and is controlled by and dependent on the presence and status of the PAs and PAIs. Plasmin activity leads to many undesirable flavour and texture defects in the product during storage.

Previous research has suggested that the plasmin in skim milk is largely unaffected by pasteurisation conditions and that, in many cases, 30–40% of its activity remains even after UHT processing. The protease system (plasminogen and its activators) is heat resistant and survives UHT treatments, but its role in the gelation of UHT-treated milk is not yet fully understood. This remains one of the long-standing unresolved problems and an important risk factor or challenge to be considered

when using casein-based ingredients in final applications, particularly neutral-pH applications.

## **1.2 COMMERCIAL SIGNIFICANCE**

Many of the neutral-pH applications of our casein-based ingredients are constrained mainly by a significant gap in capability, knowledge and understanding. The consequence of not developing this capability and knowledge will be that Fonterra might miss out on important opportunities to promote its current ingredients or new ingredients into applications in which plasmin is considered to be a risk factor.

One means of overcoming such consequences is to develop greater fundamental understanding of this problem and to develop capabilities to provide unique, cost-effective solutions. Fonterra is a leading solution provider for dairy ingredient customers by virtue of its expert knowledge in the areas of milk protein functionality and by designing tailor-made value-added ingredients for specific product applications, and would like to remain ahead in this area by continuously developing new skills, providing unique solutions to solve problems and generating new knowledge and new research capabilities. Prevention of the plasmin-induced hydrolysis of casein in the milk system is likely to provide a competitive advantage to Fonterra and will be very beneficial to Fonterra in providing solutions to this complex problem. The increase in the scientific knowledge from the project through high-quality publications and conference presentations will be beneficial to the wider group.

## **1.3 THESIS OBJECTIVES**

This proposed capability development project was aimed at addressing gaps in capability and knowledge in a systematic manner with underlying robust science. The main objectives of this research project were as follows.

Part A: Understanding the effect of substrate modification on plasmin-induced hydrolysis in a pure protein model system

- Effect of succinylation of  $\beta$ -casein on its hydrolysis by plasmin

- Effect of lactosylation on the plasmin-induced hydrolysis of  $\beta$ -casein
- Effect of transglutaminase cross-linking on plasmin-induced hydrolysis: a comparative study

Part B: Application of substrate modification in a real milk system to control plasmin-induced hydrolysis

- Succinylation of skim milk and its effect on plasmin-induced hydrolysis: effect of micellar casein structure along with substrate modification
- Plasmin resistance of high-heat-treated milk – a sequential study: effect of lactosylation and the association of whey proteins with the casein micelle on plasmin-induced hydrolysis

Part C: Understanding the plasmin-induced hydrolysis of the milk system

- Plasmin-induced dissociation of the casein micelle
- Role of plasmin in sedimentation and gelation

#### **1.4 THESIS STRUCTURE**

The thesis starts with an overall introduction, followed by a literature review and different chapters that address the above objectives. Apart from the main literature review chapter, each chapter also begins with relevant background information that is not addressed in the main literature review. The chapters describing the experimental work are divided into three sections to show the progressive approach, starting from simple systems, progressing to complex systems and concluding with the role of plasmin in sedimentation and gelation. The thesis outline is briefly described below.

Chapter 1 aims to introduce the topic of the research and starts with the problem definition and the commercial significance of the research, followed by the thesis objectives and ends with a brief outline of the whole thesis.

Chapter 2 is a general review of the literature that is relevant to the research topic and discusses different components of the complex plasmin system, the action of plasmin on different milk proteins, factors that affect plasmin activity in milk and the presence of plasmin in milk products.

The second section consists of three chapters on the prevention of plasmin-induced hydrolysis in a pure protein model system. In this section, the mechanism of the prevention of plasmin action on proteins is established using a pure protein model system and a plasmin-resistant protein is developed.

Chapter 3 describes the development of the plasmin-resistant protein using a simple chemical modification, i.e. succinylation, to establish a mechanism for the resistance of protein to plasmin.

In Chapter 4, learnings and the mechanism from the succinylation work are applied using the food-grade modification reaction, lactosylation.

The Maillard reaction is a complex reaction and involves cross-linking along with substrate modification. Therefore, the decrease in plasmin-induced hydrolysis is also studied using the food-grade cross-linking reaction, transglutamination. The effect of cross-linking using the transglutamination reaction on plasmin-induced hydrolysis is discussed in Chapter 5 and the three different means of substrate modification (succinylation, lactosylation and transglutamination) are compared to understand the mechanism better.

Chapter 6 describes the role of the structure of the casein micelle in preventing plasmin action along with the effect of succinylation on plasmin-induced hydrolysis in skim milk.

Chapter 7 describes the effect of lactosylation and the association of whey proteins with the casein micelle on plasmin-induced hydrolysis in skim milk.

Chapter 8 describes the plasmin-induced dissociation of the casein micelle and the physicochemical changes in milk as a result of plasmin action to give further insight into the role of plasmin in sedimentation and gelation in heat-treated milk.

Chapter 9 summarises the key findings of the thesis and gives recommendations.

Chapter 10 suggests future directions.

## Chapter 2: Literature review

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### 2.1 INTRODUCTION

Milk contains several indigenous enzymes that have no specific physiological role in milk. Proteases are types of enzyme that cause proteolysis; milk contains two main indigenous proteases, i.e. cathepsin D and plasmin. Plasmin is known to enter milk from the blood via mammary cell membranes. It is an alkaline serine protease and is identical to the bovine blood enzyme, plasmin (fibrinolysin, fibrinase, EC3.4.21.7<sup>1</sup>) (Benfeldt, Larsen, Rasmussen, Andreasen, & Petersen, 1995; de Rham & Andrews, 1982; Grufferty & Fox, 1988b; Kaminogawa, Mizobuchi, & Yamauchi, 1972; Korycha-Dahl, Ribadeau Dumas, Chene, & Martal, 1983; Reimerdes, 1983). Plasmin is optimally active at about pH 7.5 and 37°C (Bastian & Brown, 1996; Grufferty & Fox, 1988b). Plasmin causes the breakdown of milk proteins in a wide variety of dairy products; such hydrolysis of the milk proteins affects the flavour and the texture of dairy products and can be either beneficial or detrimental, depending on the level of activity and the type of product (Nielsen, 2002). The plasmin system in milk and its effects on milk and milk products are reviewed in detail.

The protease system in milk occurs naturally, is very complex and includes plasminogen, plasmin, plasminogen activators, plasminogen activator inhibitors and plasmin inhibitors. The complexity of the plasmin system is discussed in Section 2.2, along with a brief discussion on structural aspects and more emphasis on the mechanism of actions of each component of the plasmin system. The different components of the plasmin system, either in association with the casein micelle or in the serum phase of milk, are presented. Section 2.3 provides background information regarding the distribution of the different components in milk.

Plasmin hydrolyses different proteins in milk. Brief information on these different milk proteins is presented in Section 2.4, and the action of plasmin on these

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<sup>1</sup> Enzyme commission number.

proteins is presented in Section 2.5. Different factors influencing the activity of the plasmin system in milk are briefly discussed in Section 2.6. The measurement of plasmin activity and the presence of plasmin in different milk products are discussed in Sections 2.7 and 2.8 respectively.

## **2.2 THE PLASMIN SYSTEM**

Plasmin is part of a complex protease–protease inhibitor system in milk, as shown in Figure 1, consisting of plasmin, its inactive form plasminogen, plasminogen activator, which converts plasminogen (786 residues; molecular mass 88,092 Da) to plasmin by cleavage of an Arg–Ile bond at position 557–558 of the plasminogen molecule (Bastian & Brown, 1996; Grufferty & Fox, 1988d; Schaller et al., 1985), plasminogen activator inhibitor, which inhibits plasminogen activator activity, and plasmin inhibitor, which inhibits plasmin activity, depending on the processing conditions (Bastian & Brown, 1996; Precetti, Oria, & Nielsen, 1997; Richardson, 1983a).

Plasmin, plasminogen and plasminogen activators are predominantly bound to the casein micelle in milk, whereas plasmin inhibitors are located in the milk serum (Baer, Ryba, & Collin, 1994; de Rham & Andrews, 1982; Korycha-Dahl et al., 1983; Richardson, 1983a, 1983b). The presence of plasmin in the milk fat globule membrane (Hofmann, Keenan, & Eigel, 1979) is due to contamination with the casein micelle (Benfeldt et al., 1995; Politis, Barbano, & Gorewit, 1992).

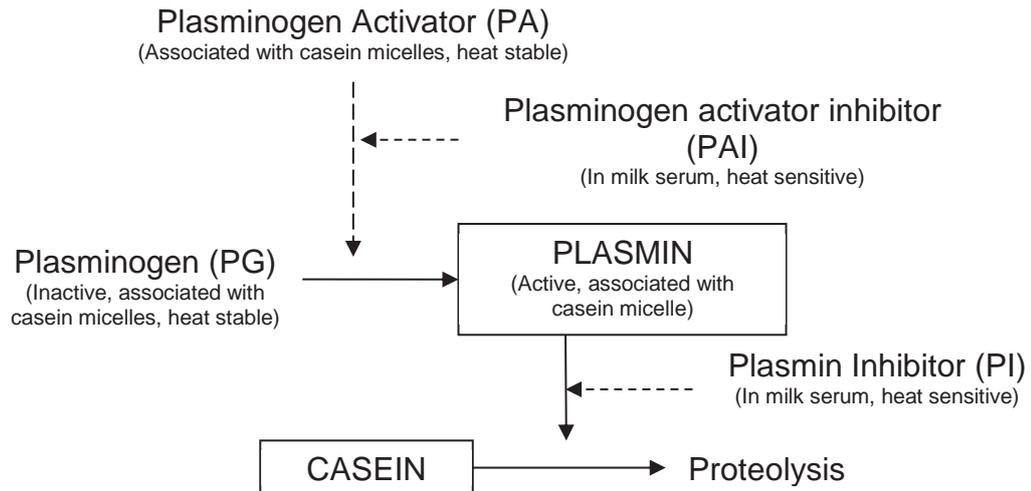


Figure 1. Plasmin system in bovine milk. Compiled from Ismail and Nielsen (2010), Precetti et al. (1997), Reimerdes and Klostermeyer (1974) and Richardson (1983a).

Plasmin isolated from milk and plasmin isolated from blood are identical, as indicated by similar pH optima and stabilities, heat stabilities, casein hydrolytic specificities and inhibition patterns (Kaminogawa et al., 1972; Reimerdes, 1983). Benfeldt et al. (1995) sequenced 15% of the amino acids in bovine milk plasminogen and found them to be identical to those in bovine blood plasminogen (Bastian & Brown, 1996). Plasmin's activity and characteristics are similar to those of trypsin (same alkaline serine protease family), with plasmin having more specificity (Eigel et al., 1979; Fox, 1981b; Korycha-Dahl et al., 1983; Reimerdes, 1981; Richardson & Pearce, 1981). Both plasmin and trypsin hydrolyse proteins on the carboxyl site of Lys-X and Arg-X bonds, with a preference for Lys-X bonds (Kitchen, 1985). Like trypsin, plasmin is inhibited by diisopropyl fluorophosphate, soya bean trypsin inhibitor and tosyllysine chloromethyl ketone (Kaminogawa et al., 1972).

Plasmin enters milk from blood in its inactive zymogen form, plasminogen, via the mammary cell wall lining; the concentrations of plasmin in milk and blood are ~ 0.3 and 200 mg/mL respectively (Halpaap, Reimerdes, & Klostermeyer, 1977). Both plasmin and plasminogen are associated mainly with the casein fractions of milk (de Rham & Andrews, 1982; Korycha-Dahl et al., 1983; Reimerdes & Klostermeyer, 1974; Richardson, 1983a). In fresh milk, the plasminogen concentration is 0.55–2.75

µg/mL, which is 2–30 times the plasmin concentration – 0.14–0.73 µg/mL (Richardson & Pearce, 1981). The activity of plasmin in milk depends significantly on the activation of plasminogen. The activation of plasminogen into plasmin by plasminogen activator can occur while the milk is in the mammary lumen, before milking, and during milk storage (Alichanidis, Wrathall, & Andrews, 1986; de Rham & Andrews, 1982; Driessen & van der Waals, 1978; Schaar, 1985).

### 2.2.1 Plasmin and plasminogen

#### **Structure**

Bovine plasminogen is a glycoprotein that contains 786 amino acids and, based on its amino acid sequence and carbohydrate content, has a molecular mass of 88,092 Da (Benfeldt et al., 1995; Schaller et al., 1985). From the amino-terminal (N-terminal) residue, Asp1 of mature plasminogen, the first 77 residues are known as the pre-activation peptide (PAP). The PAP is followed by five consecutive characteristic triple-loop structures, called 'kringles' [the sequence structure looks like the Danish pastry kringle (Williamson, 2012)], as shown in Figure 2. Each kringle is stabilised by three intramolecular disulphide bonds; plasminogen has a total of 24 disulphide bonds (Fox, 2002; Schaller et al., 1985). The kringle domains are: K1, Cys84–Cys162; K2, Cys166–Cys243; K3, Cys256–Cys333; K4, Cys358–Cys435; K5, Cys459–Cys538 (Schaller et al., 1985). The kringle domains interact with lysine-like ligands and facilitate the binding of plasminogen to large substrates, e.g. fibrin, caseins etc., and to small molecules, e.g.  $\alpha$ - and  $\epsilon$ -amino acids,  $\text{Cl}^-$  etc. (Urano, Chibber, & Castellino, 1987a; Urano, Sator de Serrano, Chibber, & Castellino, 1987b), through lysine-binding sites. These lysine-binding sites play a very important role in the activation of plasminogen to plasmin. They are present on kringles; one kringle has higher affinity for lysine than the other four kringles (Bastian & Brown, 1996). The five kringle series is attached to the functional serine protease domain (Ile558–Asn786), which becomes activated upon the catalytic cleavage of a single peptide bond, Arg557–Ile558, by the action of plasminogen activator (Schaller et al., 1985).

Schaller et al. (1985) identified the complete amino acid sequence of bovine plasminogen, which is presented in the schematic in Figure 2, showing the primary structure of plasmin. Bovine plasminogen and human plasminogen are assumed to have many similar features (functional and structural regions) because of their almost 78% similar amino acid sequences and identical heavy and light chain regions (Schaller et al., 1985). Bovine plasminogen comprises 786 residues compared with 791 residues for human plasminogen. The five kringle structure present in bovine plasminogen is identical to that in human plasminogen, because of the identical presence and distribution pattern of half-cysteine residues in the heavy chain of bovine plasminogen (Schaller et al., 1985). Bovine plasminogen also contains two carbohydrate moieties. The N-glycosidic site in bovine plasminogen, Asn289, is localised in kringle 3 compared with Asn288 in human plasminogen, whereas the O-linked carbohydrate attachment site in the human sequence, Thr345, is shifted to Ser339 in bovine plasminogen (Marti et al., 1988; Schaller et al., 1985).

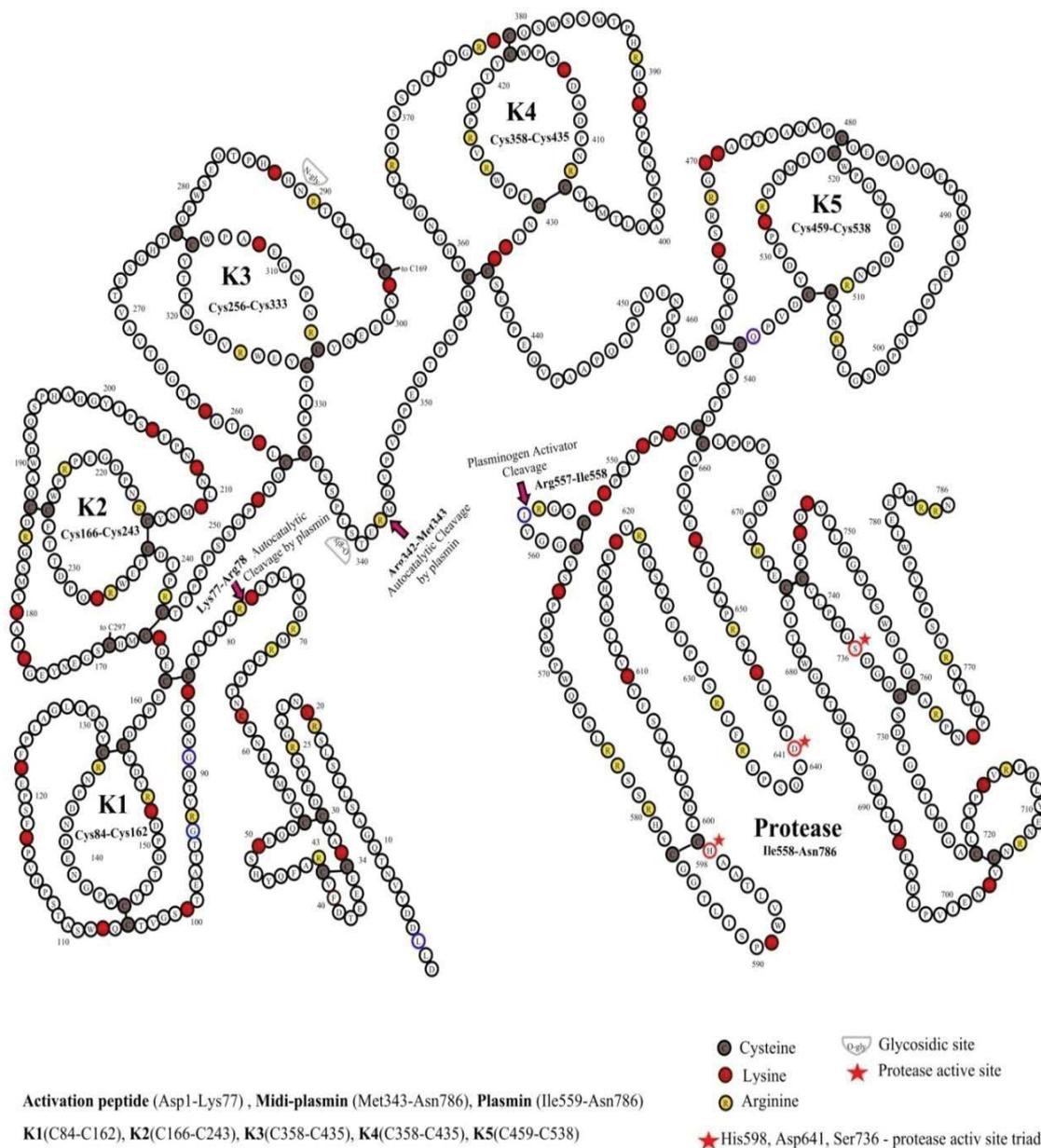


Figure 2. The structure of bovine plasminogen. Complete amino acid sequence of bovine plasminogen, the catalytic triad of the protease (plasmin), the disulphide (–) arrangement, plasmin cleavage sites to release the PAP fragment and the activation cleavage site (plasminogen activator attack) are drawn from Benfeldt et al. (1995) and Schaller et al. (1985). The autoproteolysis cleavage site to release midi-plasmin(ogen) is drawn from Benfeldt et al. (1995). Redrawn from Benfeldt et al. (1995), Castellino and Ploplis (2003), Kelly and McSweeney (2003), Schaller et al. (1985) and UniProtKB (2011).

The major functional difference between bovine plasminogen and human plasminogen is in their ability to be activated into plasmin by streptokinase (Schaller et al., 1985). A trace amount of streptokinase is sufficient for the activation of

human plasminogen (Wulf & Mertz, 1969), whereas streptokinase has no activating effect in bovine plasminogen. This could be because of the absence of a streptokinase interaction site in bovine plasminogen (reason not known), which is located in the light chain in human plasmin(ogen) (Schaller et al., 1985).

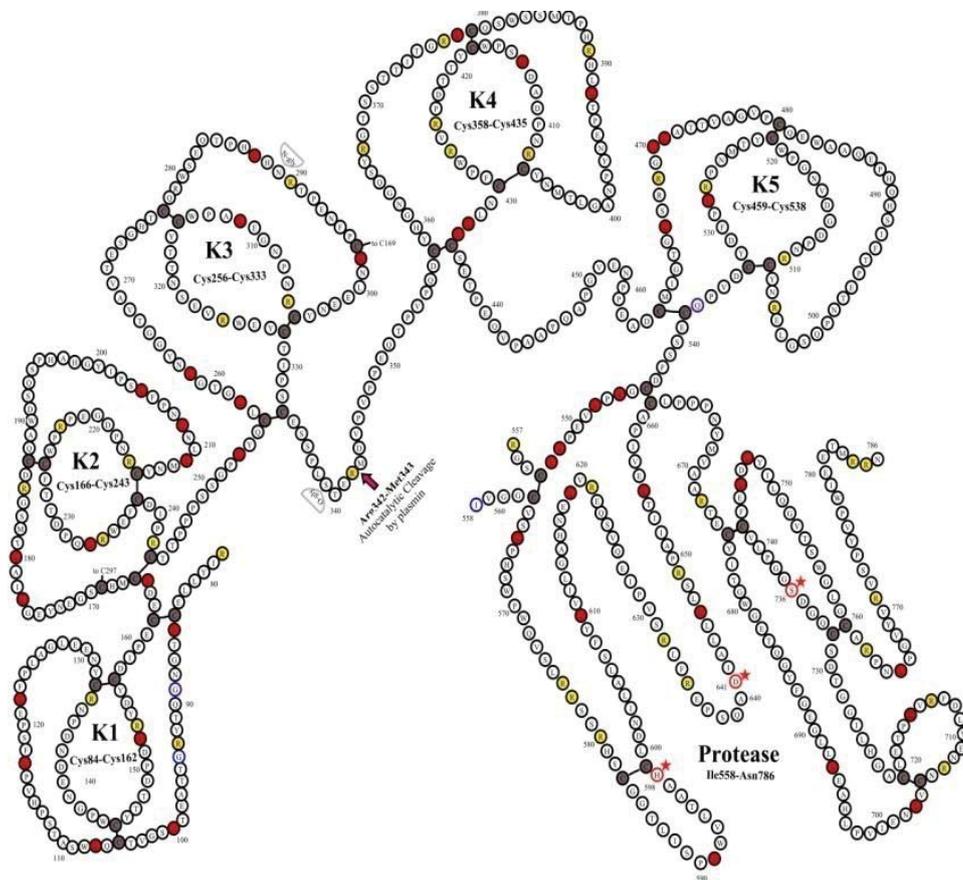


Figure 3. Plasmin, generated by the release of the PAP and catalytic cleavage between Arg557 and Ile558 by plasminogen activators. The resulting activated plasmin consists of two disulphide-linked polypeptide chains: the heavy chain (five kringles) and the light chain (protease domain).

Plasmin is a glycoprotein that exists as a dimer that is held together by disulphide bonds (Figure 3). It has a molecular mass of 48,000 Da, as determined by size exclusion chromatography (Kaminogawa et al., 1972; Nielsen, 2003); much higher molecular weights have also been reported. Partial unfolding (e.g. by heat treatment) readily facilitates the autodigestion of plasmin (Metwalli, de Jongh, & van Boekel, 1998). Bovine plasmin is similar to human plasmin; both consist of two polypeptide chains – the heavy chain (derived from the amino-terminal region) and the light chain (originating from the carboxyl terminus) (Robbins, Summaria, Hsieh,

& Shah, 1967) – linked by two interchain disulphide linkages (Grufferty & Fox, 1988b). The heavy chain contains five kringles, in which lysine-binding sites are located, and the light chain consists of the serine protease domain (similar to trypsin) (Benfeldt et al., 1995; Wiman, 1977; Wiman & Wallén, 1975). Benfeldt et al. (1995) found a truncated form, consisting of kringle 4, kringle 5 and the serine protease domain, which was generated through further hydrolysis of plasmin at Arg342–Met343 (Benfeldt et al., 1995); they named it midi-plasmin (Christensen, Sottrup-Jensen, & Christensen, 1995a), as shown in Figure 4.

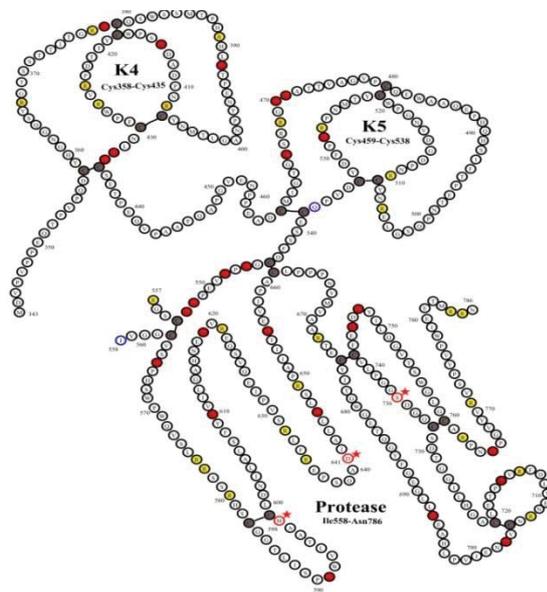


Figure 4. Midi-plasmin model with the complete amino acid sequence (midi-plasmin contains kringle 4, kringle 5 and the serine protease domain).

Benfeldt et al. (1995) observed one band of 50 kDa midi-plasmin on non-reduced sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels and two separate fragments as bands of 30 and 26 kDa from midi-plasmin on reduced SDS-PAGE gels (Benfeldt et al., 1995).

### ***Activation of bovine plasminogen***

The bovine plasminogen molecule (Figure 2), consisting of 786 amino acid residues (molecular weight 88,092 Da), is activated through catalytic cleavage at the Arg557–Ile558 bond activation site by plasminogen activators (urokinase or tissue plasminogen activator) as well as through autocatalytic cleavage at Lys77–Arg78,

releasing the PAP (pre-activation peptide) fragment (Figure 3) (Benfeldt et al., 1995; Schaller et al., 1985). Autocatalytic cleavage of bovine plasminogen by plasmin at Lys77–Arg78 releases an N-terminal PAP and results in the formation of a mature enzyme, Arg-plasmin(ogen) (inactive), of 81 kDa containing five kringles and a serine protease domain (Benfeldt et al., 1995). The release of the PAP causes a conformational change in plasminogen, making it easier for plasminogen activators to activate it into plasmin (Figure 5). Then, Arg557–Ile558 is cleaved by urokinase or tissue plasminogen activator to give a mature enzyme of ~ 81 kDa; it is an active two-chain molecule, consisting of a heavy chain and a light chain that are joined by two disulphide bonds (Benfeldt, Sørensen, & Petersen, 1998; Fox, 1981b). The active site of bovine plasmin is in the light chain and, like the active site of trypsin, contains His598, Asp641 and Ser736 (Schaller et al., 1985).

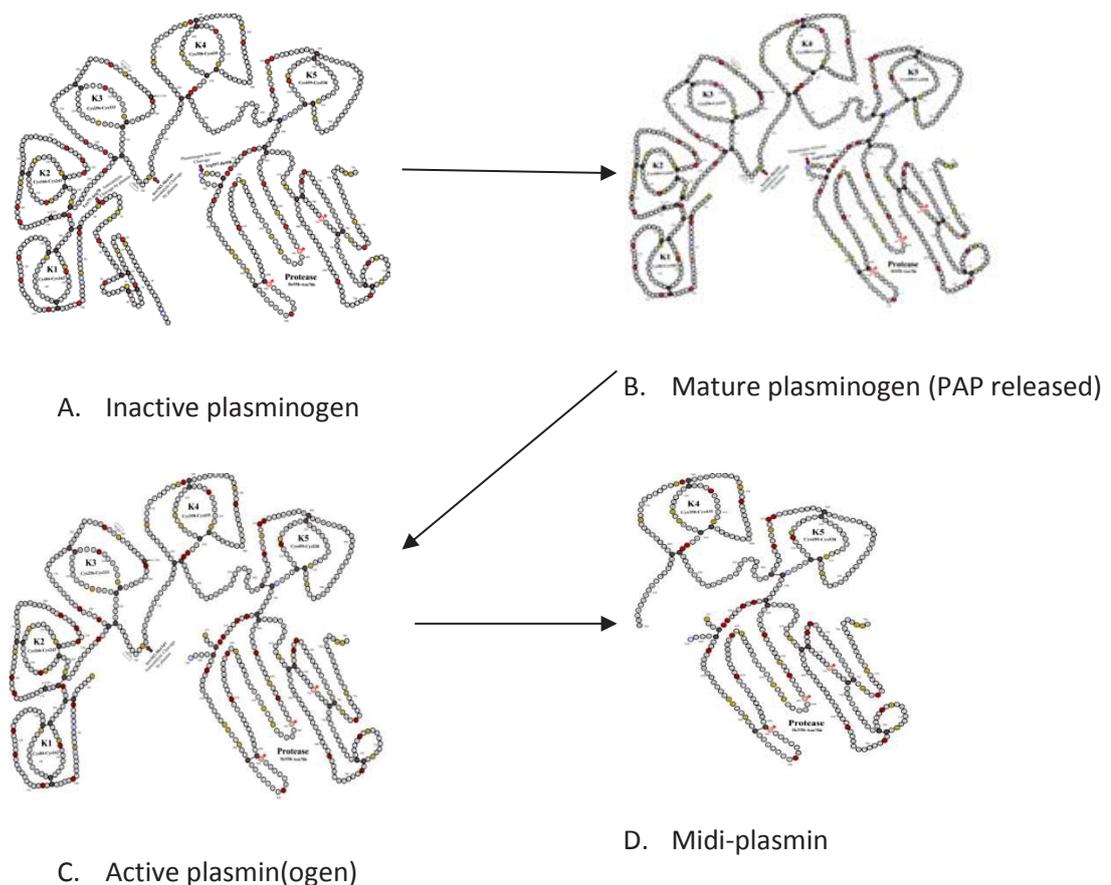


Figure 5. Plasminogen activation: A, plasminogen (inactive); B, mature plasminogen (release of PAP); C, active plasmin(ogen), Arg557–Ile558 hydrolysis by plasminogen activators; D, midi-plasmin, Arg342–Met343 hydrolysis by plasmin.

The active two-chain molecule is further hydrolysed at the Arg342–Met343 bond by plasmin and results in the truncated form called midi-plasmin (Christensen et al., 1995a) consisting of kringle 4, kringle 5 and the serine protease domain (Benfeldt et al., 1995). The corresponding bond for generating midi-plasmin in human plasminogen is Glu343–Leu344, which is not hydrolysed by plasmin (Benfeldt et al., 1995). Once conversion of plasminogen to plasmin has been initiated, plasmin can promote further plasminogen conversion autocatalytically (Saksela & Rifkin, 1988), and can readily autodigest if it has been partially unfolded (e.g. by heat treatment) (Nielsen, 2003).

### ***Substrate specificity and hydrolysis mechanism of protease***

Plasminogen contains five kringle domains, which have lysine-binding sites (Baer et al., 1994; Benfeldt et al., 1995). Plasminogen adopts a closed conformation in the absence of fibrin (or lysine analogue) and becomes open when bound to fibrin. As the open structure has a much higher activation rate, the kringle domain interactions control the overall conformation of plasminogen and ultimately provide the site specificity of activation (Wang et al., 2000). The activation of bovine plasminogen by activators occurs by limited proteolysis of peptide bonds, resulting in the formation of active plasmin. Nagasawa and Suzuki (1970) showed that the N-terminal sequence of the light chain of bovine plasmin is homologous to that of bovine trypsin. The N-terminal sequence of bovine plasminogen has been reported to be Asp–Ile(Leu)–Ile(Leu)–Asp, but becomes Ile–Val–Gly–Gly after its activation by urokinase. This is an essential step for the formation of functionally active bovine plasmin, i.e. the cleavage of a peptide bond involving an isoleucine, so that isoleucine becomes the N terminus of the light chain (as observed for bovine trypsin, chymotrypsin and bovine thrombin) (Nagasawa & Suzuki, 1970).

Plasminogen, unlike other serine proteases, is structurally restrained by a disulphide bond (human plasminogen, Cys558–Cys566; bovine plasminogen, Cys554–Cys662) on its proteolytic activation cleavage site, which brings unique properties to plasminogen activation (Wang et al., 2000). The rates of peptide hydrolysis by serine proteases are  $\sim 10^{10}$  fold higher than the uncatalysed reaction rates

(Hedstrom, 2002) because three main hurdles in peptide bond hydrolysis are overcome: (a) amide bonds are very stable because of electron donation from the amide nitrogen to the carbonyl; proteases activate an amide bond through the carbonyl oxygen and also distort the peptide bond to disrupt the resonance stabilisation; (b) water is a poor nucleophile; proteases always activate water, usually via a general base; (c) amines are poor leaving groups; proteases protonate the amine prior to expulsion (Hedstrom, 2002).

### *Catalytic triad*

Plasmin system enzymes – plasmin, urokinase and tissue plasminogen activator – belong to the trypsin-like serine protease family (Polgár, 2005). Serine proteases are named for their nucleophilic serine residue (the serine hydroxyl group is an essential catalytic group) at the active site (Hedstrom, 2002). The active site serine residue is assisted by histidine and an aspartate residue and is known as the ‘catalytic triad’ or ‘charge relay’ system (Figure 6); it is located and preserved in an active site of serine proteases and plays an essential role in proteolysis (Di Cera, 2009; Perona & Craik, 1995; Polgár, 2005). The serine proteases hydrolyse both peptide and ester bonds of substrates; the hydrolysis of ester bonds is much faster than that of peptide bonds ( $\Delta H^*$  is  $\sim 7$  kcal/mol for ester bonds versus 20 kcal/mol for amide bonds) (Whitaker, 2002).

Any change in the catalytic triad residues or the spatial relationship markedly changes the enzyme specificity and the hydrolysis rate, e.g. (a) substitution of Asp102 of trypsin prevents His57 from being able to accept a proton from Ser195 in trypsin; (b) a change in His603 influences the nucleophilic character of Ser741 in human plasminogen (Craik, Rocznik, Largman, & Rutter, 1987; Mhashilkar, Viswanatha, Chibber, & Castellino, 1993; Polgár, 2005).

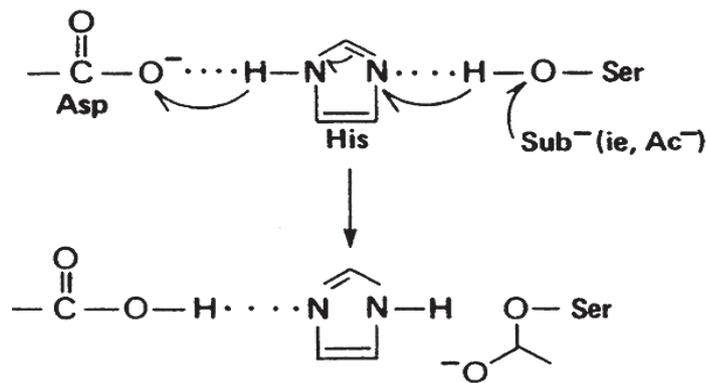


Figure 6. Catalytic triad of serine protease.

The catalytic domain contains conserved residues that form a catalytic triad, i.e. His598, Asp641 and Ser736 in bovine plasmin(ogen) and His57, Asp102 and Ser195 in trypsin (Schaller et al., 1985; Sheehan & O'Sullivan, 2006). Each residue of the catalytic triad plays a specific role in proteolysis. The serine residue acts as a nucleophile and attacks at the carbonyl carbon of a scissile peptide bond, histidine acts as a base and makes serine more nucleophilic, and the carboxyl of aspartic acid forms hydrogen bonds with histidine, making it more electronegative (Hedstrom, 2002).

#### *Zymogen activation domain*

Plasminogen, like most serine protease zymogens, is an inactive precursor. Its activation by plasminogen activator releases the N-terminal Ile558, which is Ile16 in trypsin (Benfeldt et al., 1995; Hedstrom, 2002). The new N terminus forms a buried (solvent-inaccessible) salt bridge bond with Asp194 in trypsin and Asp740 in human plasmin, inducing a conformational change that orders the activation domain (Hedstrom, 2002; Wang et al., 2000). The specificity pocket and the oxyanion hole are formed, creating the active protease of plasmin (Hedstrom, 2002; Wang et al., 2000). At higher pH, the N terminus becomes deprotonated and disruption of the Ile16–Asp194 salt bridge occurs, leading to a shift in the conformational equilibrium towards an inactive zymogen-like conformation and ultimately loss of protease activity (Hedstrom, 2002).

### *Substrate recognition sites, specificity pocket (S1) and oxyanion hole*

The substrate recognition sites include the polypeptide binding site and the binding pockets for the side chains of the peptide substrate. The specificity pocket S1 is the substrate-binding site (of an enzyme) on the protease that attaches to P1 (peptide residues) of the substrate and forms a P1–S1 interaction (Hedstrom, 2002).

The S1 site is a pocket adjacent to Ser195 in serine proteases and the specificity of the serine protease is driven by a residue located at the bottom of the enzyme's S1 pocket (Perona & Craik, 1995). The side chain of Asp735 in human plasmin and of Asp189 in trypsin (Figure 7) is located at the bottom of the S1 site, which creates a negatively charged S1 site and accounts for the specificity for positively charged P1 residues of the substrate, especially lysine or arginine (Krieger, Kay, & Stroud, 1974; Wang et al., 2000). According to Perona and Craik (1995), the substrate-binding affinity depends upon the accessibility of the negative charge to the substrate and not upon the formation of direct interactions with protease. The interactions of arginine and lysine substrates with the primary determinant aspartic acid (in the specificity pocket) are different; the positively charged guanidinium group of P1–Arg substrates forms an ion-pair interaction, whereas the positively charged ammonium of P1–Lys forms a water-mediated contact; both form salt linkages with the negatively charged carboxylate of aspartic acid (Fersht, 1999; Hedstrom, 2002).

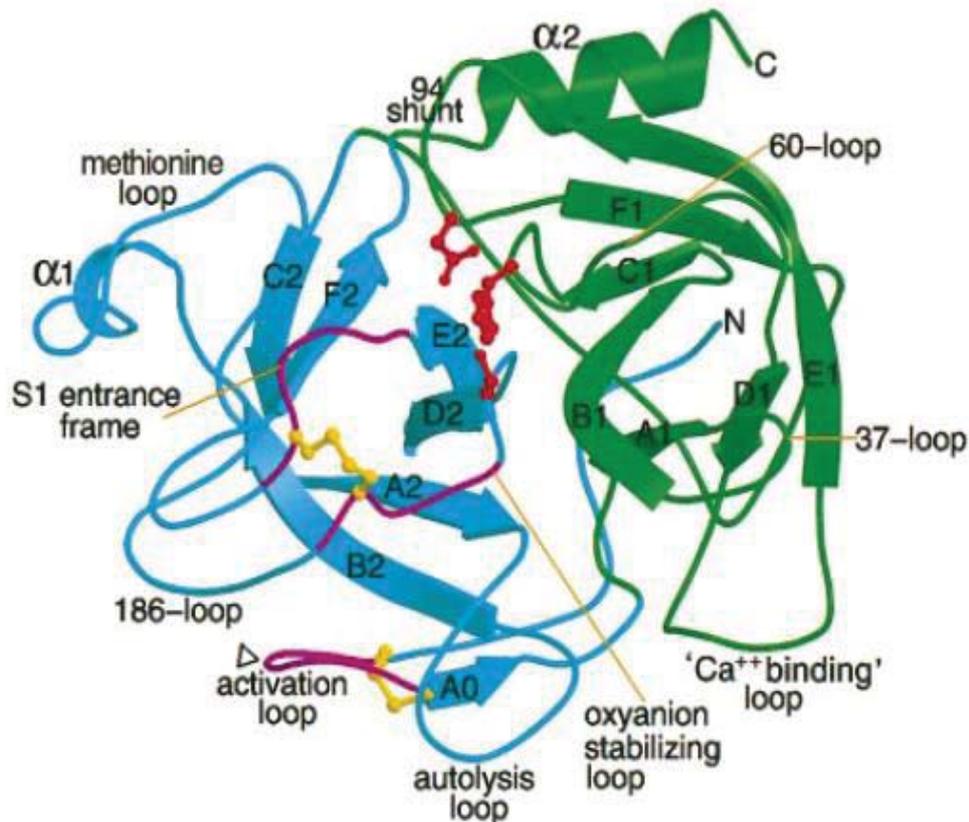


Figure 7. Substrate-binding pocket (substrate-binding sites) in a serine protease, i.e. human-plasmin (Wang et. al., 2000).

The aspartic acid in the specificity pocket is critical for the serine protease trypsin (similar to plasmin) in two ways: (a) it provides tight binding affinity; (b) it has a high acylation rate; therefore, the precise location of the negatively charged group within the specificity pocket is critical to positioning the scissile bond in the catalytic register with the serine and histidine of a catalytic triad (Perona & Craik, 1995).

In the serine protease trypsin, the oxyanion hole is formed by the backbone hydrogen atoms of Gly193 and Ser195; it is a pocket of positive charge and activates the carbonyl of the scissile peptide bond and stabilises the negatively charged oxyanion of the tetrahedral intermediate (Hedstrom, 2002; Perona & Craik, 1995).

#### *Mechanism of the serine protease plasmin*

The protein substrate binds to the surface of plasmin such that the side chain amino group of lysine or arginine residues enters the specificity pocket (S1) by the

attraction of Asp189 and the carbon ion of the scissile bond becomes positioned near the nucleophilic serine. The catalytic triad is His57, Asp102 and Ser195 in the serine protease trypsin and is His598, Asp641 and Ser736 in bovine plasminogen (Schaller et al., 1985).

Steps of the serine protease mechanism:

**1. Attack by serine:**

- The hydroxyl oxygen of Ser195 loses its hydrogen to His57 and becomes more nucleophilic (Step 1).
- The nucleophilic oxygen attacks the carbonyl of the scissile bond, the pair of electrons from the carbonyl oxygen moves to the oxygen and a tetrahedral intermediate is formed (Step 2).
- The resulting tetrahedral geometry is stabilised by two amide hydrogen atoms of Gly193 and Ser195, coordinating the anionic oxygen (which occupied the 'oxyanion hole') (Step 3).

**2. The acyl-enzyme:**

- The tetrahedral intermediate decomposes to an acyl-enzyme intermediate (Step 4).
- The peptide bond is broken, and the N-terminal portion of the substrate diffuses away.
- The remaining substrate is temporarily covalently linked to the enzyme (Step 5).

**3. Attack by water:**

- A water molecule enters and attacks the ester bond linking the substrate and the enzyme (Step 6).
- A tetrahedral intermediate forms again as one of the hydrogens of the water is passed to His57 (Step 7).

**4. Hydrolysis:**

- The tetrahedral intermediate decomposes to free the substrate (Step 8).
- Ser195 recovers its hydrogen from His57, the C-terminal portion of the substrate diffuses out of the active site and the enzyme is restored to its initial state.

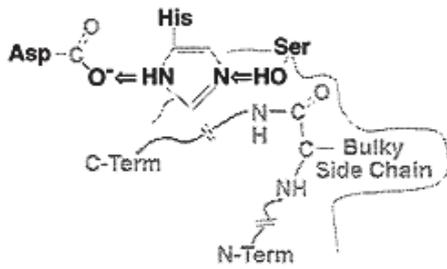
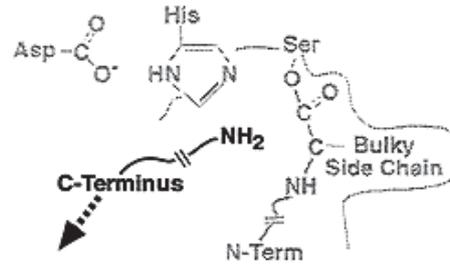
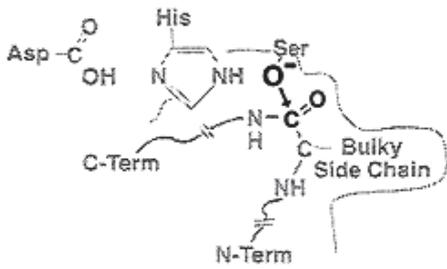
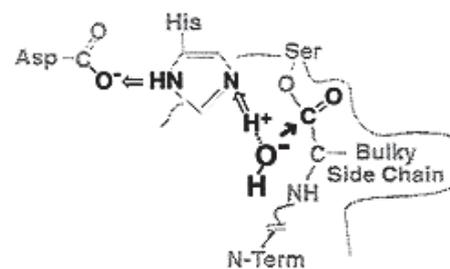
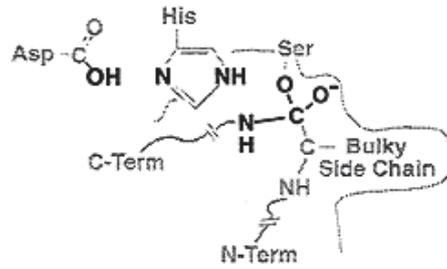
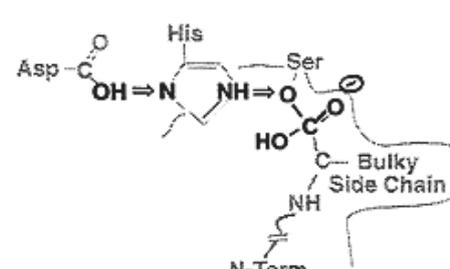
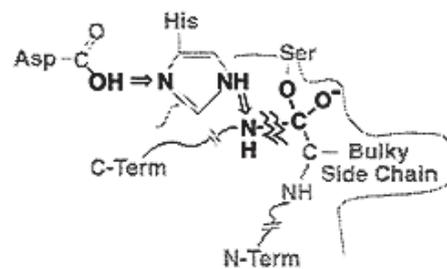
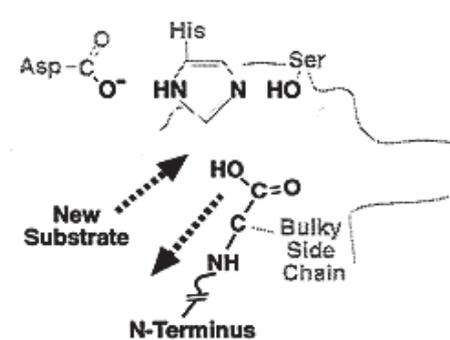
**Step 1: H<sup>+</sup> shift generates Ser-O<sup>-</sup>****Step 5: C-Terminal peptide leaves****Step 2: Ser-O<sup>-</sup> binds to C=O****Step 6: ionization of water****Step 3: Transition State I****Step 7: Transition State II****Step 4: Peptide bond breaks****Step 8: N-terminal peptide leaves**

Figure 8. Serine protease mechanism (copied and modified from [http://chemistry.gravitywaves.com/CHE450/11\\_Enz.%20Mech.-Ser%20Protea.htm](http://chemistry.gravitywaves.com/CHE450/11_Enz.%20Mech.-Ser%20Protea.htm)).

## 2.2.2 Plasminogen activators

Plasminogen is converted into plasmin by a group of enzymes called (serine protease) plasminogen activators (PAs). A PA activates plasminogen to plasmin by

cleaving an Arg557–Ile558 bond in bovine blood plasminogen (Driessen & van der Waals, 1978; Schaar, 1985; Schaller et al., 1985) and an Arg560–Val561 bond in human plasminogen (Robbins et al., 1967). Deharveng and Nielsen (1991) found at least four native PAs in bovine milk. Those that are indigenous to milk are divided into two main classes. Two structurally similar, but functionally and immunologically distinct, serine proteases effectively activate plasminogen (Saksela & Rifkin, 1988): urokinase-type (uPA) (EC 3.4.21.73) and tissue-type (tPA) (EC 3.4.21.68). Researchers have shown the presence of tPA and uPA activators (Deharveng & Nielsen, 1991) in bovine mammary tissue (Heegaard, White, Zavizion, Turner, & Politis, 1994b) and in milk (Heegaard, Rasmussen, & Andreasen, 1994a; Lu & Nielsen, 1993a; Politis, Zhao, McBride, Burton, & Turner, 1991; White et al., 1995). The interaction of these PAs with plasminogen is regulated at several levels. The modulation of cellular PA synthesis directly controls extracellular PA activity (Saksela & Rifkin, 1988). The associations of the PAs are different; tPA is associated mainly with casein, whereas uPA is associated with somatic cells, interacting with a uPA receptor (Heegaard et al., 1994a; White et al., 1995). Molecular masses of 93, 57, 42, 35 and 27 kDa were reported for bovine milk PAs (the presence of five proteins capable of activating plasminogen), presumably uPA, using the casein-plasminogen SDS-PAGE technique (Lu & Nielsen, 1993c).

PAs are more heat stable than plasmin and plasminogen, as observed by Lu and Nielsen (1993b). The decimal reduction times (D values) of PAs were found to be 109 min at 70°C and 32 s at 140°C (Lu & Nielsen, 1993b), indicating that the PAs in bovine milk are not affected by a pasteurisation process and are largely not inactivated by ultra-high-temperature (UHT) dairy processing conditions (Lu & Nielsen, 1993b). In contrast, PA inhibitor does not survive pasteurisation temperatures (Prado, Sombers, Ismail, & Hayes, 2006; Richardson, 1983a), which means that PAs can have a significant effect on plasmin activity in milk systems and this leads to proteolysis in milk products.

The amounts of uPA and tPA, their activities and their molecular weights depend on and are varied by the nature of the milk, the methods of separation and the assay methods used (Ismail, Choi, Were, & Nielsen, 2006). It has been reported that tPA is

the major PA in human milk and in bovine milk somatic cells (Zachos, Politis, Gorewit, & Barbano, 1992). This differs from reports that state that the predominant PA in human skim milk, in extracts of bovine casein micelles and in human and bovine cultured milk macrophages is of the uPA type (Deharveng & Nielsen, 1991; Ismail et al., 2006; Politis et al., 1991). The activities of both tPA and uPA are stimulated by casein (Markus, Hitt, Harvey, & Tritsch, 1993). uPA is more thermally stable than tPA (Prado, Ismail, Ramos, & Hayes, 2007) and a very small amount of uPA can cause more activation of plasminogen than a large amount of tPA (Ismail et al., 2006); further, tPA requires the presence of fibrin (or fibrin-like compound) (Karlan, Clark, & Littlefield, 1987), which indicates that uPA plays a more significant role than tPA in the activation of plasminogen during the storage of heated milk (Lu & Nielsen, 1993c).

Up to 75% homology between bovine PA and human PA (Heegaard et al., 1994b) means that human PAs are useful (accelerated cheese ripening) (Barrett, Kelly, McSweeney, & Fox, 1999; Bastian, Hansen, & Brown, 1991b; Bastian, Lo, & David, 1997) and are often used in model studies in place of bovine PAs (Wang, Hayes, & Mauer, 2007). The main physiological function of tPA is in thrombolysis, whereas uPA is involved in tissue-remodelling events (Politis, 1996). Unlike human plasminogen, bovine plasminogen cannot be activated by streptokinase, because of the absence of a streptokinase interaction site in bovine plasminogen (Schaar, 1985). The difference in specificity of streptokinase and uPA is attributed to the fact that streptokinase forms a complex, which is the activator, whereas uPA itself is capable of cleaving the single peptide bond required for the activation of plasminogen (Wulf & Mertz, 1969).

### ***Urokinase-type PA***

uPA is one of the main PAs present indigenously in the milk system. According to recent studies, uPA is synthesised and released from mammalian cells as a single-chain inactive zymogen (pro-uPA) with little or no activity (Conese & Blasi, 1995). As the same cells can also synthesise inhibitors of PAs, the location and the extent of plasminogen activation is regulated by the activation of pro-uPA, the uPA receptor

occupancy and PA inhibitors (Blasi, Vassalli, & Dano, 1987). Plasmin and proteases of other classes such cathepsin B, cathepsin L (Goretzki et al., 1992) or kallikrein (Stoppelli, 2000) cause the activation of pro-uPA (inactive) to two-chain uPA (active), which is accelerated in uPA-receptor-bound uPA (Conese & Blasi, 1995).

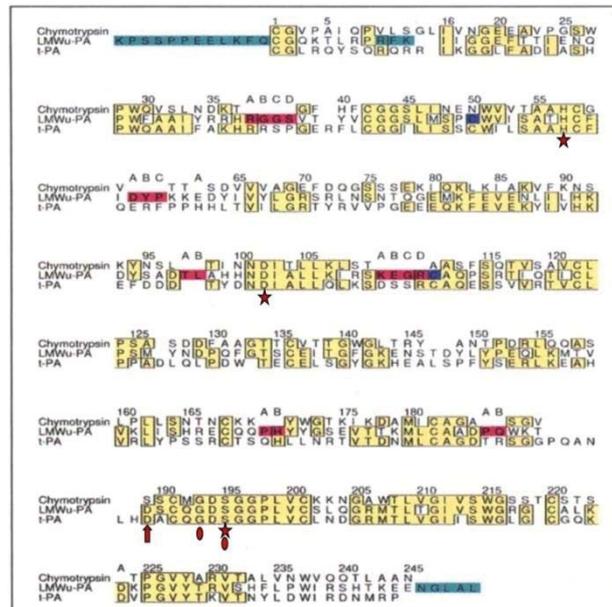


Figure 9. Sequence similarity of uPA and tPA proteases with chymotrypsin. Catalytic triad residues are indicated by stars, the arrow indicates the substrate specificity pocket of Asp and ovals indicate the oxyanion-hole-forming residues. The first two rows show a structural alignment between chymotrypsin and low molecular weight (LMW) uPA: structurally variable loops are shown in red and conserved residues are shown in yellow. Residues involved in the additional disulphide bridge in LMW uPA are highlighted in blue; residues that are not fitted to the electron density map are highlighted in green. The sequence of tPA serine protease is shown in the third row, based on a sequence alignment with LMW uPA. It shows 46% sequence identity to LMW uPA. Copied and modified from Spraggon et al. (1995).

Human pro-uPA (single-chain uPA; 54 kDa) consists of 411 amino acids with 12 disulphide bonds (Bi, Cen, Huang, & Zhu, 2002; Stepanova & Tkachuk, 2002). Plasmin cleaves the Lys158–Ile159 peptide bond (Figure 10), generating uPA, a two-chain (light chain A and heavy chain B) molecule that is held together by a single (Cys148–Cys279) disulphide bond; the light chain includes the growth factor (epidermal-growth-factor-like module) and kringle domains, whereas the heavy chain consists of the protease domain (Lijnen, Nelles, Holmes, & Collen, 1988; Stepanova & Tkachuk, 2002; Stoppelli, 2000). The newly formed light chain

undergoes a conformational change and Ile159 is relocated into the substrate-binding pocket of the protease and forms an ion pair between the  $\text{NH}_2^-$  group of Ile159 and Asp255, ultimately resulting in an opening of the substrate-binding pocket and the active site of the enzyme (Stepanova & Tkachuk, 2002).

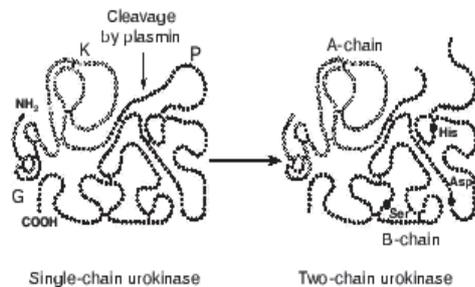


Figure 10. uPA. A-chain, growth factor and kringle domain; B-chain, protease domain with His204, Asp255 and Ser256 (Castellino & Ploplis, 2003), the catalytic triad of the serine protease [adopted from Stepanova & Tkachuk (2002)].

Each domain of uPA is supported by internal disulphide bonds (three in the growth factor domain, three in the kringle domain and six in the protease domain). The destruction or the irregular formation of intramolecular disulphide bonds results in loss of enzymatic activity; this indicates the importance of intramolecular disulphide bonds in maintaining the orientation of amino acids in the protease domain of uPA (Stepanova & Tkachuk, 2002). The active site catalytic triad consists of His204, Asp255 and Ser356 (Dobrovolsky & Titaeva, 2002).

A third form of uPA was found to be derived from proteolytic cleavage at the Lys135–Lys136 peptide bond by plasmin or uPA itself, with less sensitivity (Lijnen et al., 1988; Mangel, Lin, & Ramakrishnan, 1991). This form of uPA is termed low molecular weight uPA and is the major form found in urine (Ellis, 2003). uPA-receptor-bound uPA is susceptible to inhibition by its specific inhibitors (PAI-1, PAI-2 and protease nexin 1) (Conese & Blasi, 1995). Although pro-uPA (single-chain form) can convert plasminogen into plasmin, the two-chain uPA cleaves plasminogen 200-fold faster than the single-chain form (Stepanova & Tkachuk, 2002). Politis et al. (1991) did work on the production and secretion of a PA protein by bovine monocytes and macrophages, and documented the production of a 28 kDa PA

protein with similar characteristics to uPA (activity not affected by the presence of fibrin) (Politis et al., 1991).

### ***Tissue-type plasminogen activator***

tPA is a serine protease of 68 kDa that is synthesised as a 527-amino-acid single-chain molecule (Castellino & Ploplis, 2003). The two-chain form (disulphide linked) of tPA is derived by plasmin cleavage at the Arg275–Ile276 peptide bond and has 10–50-fold increased activity compared with the single-chain form (Figure 11) (Castellino & Ploplis, 2003; Stoppelli, 2000). The N-terminal-derived heavy chain (A) contains a finger domain (fibronectin-type II), an epidermal-growth-factor-like domain and two kringle domains (K1 and K2) (Politis, 1996). The light chain (B) contains a large catalytic domain (homologous to uPA and other serine proteases) and has His322, Asp371 and Ser 478 as the catalytic triad (Castellino & Ploplis, 2003; Stoppelli, 2000).

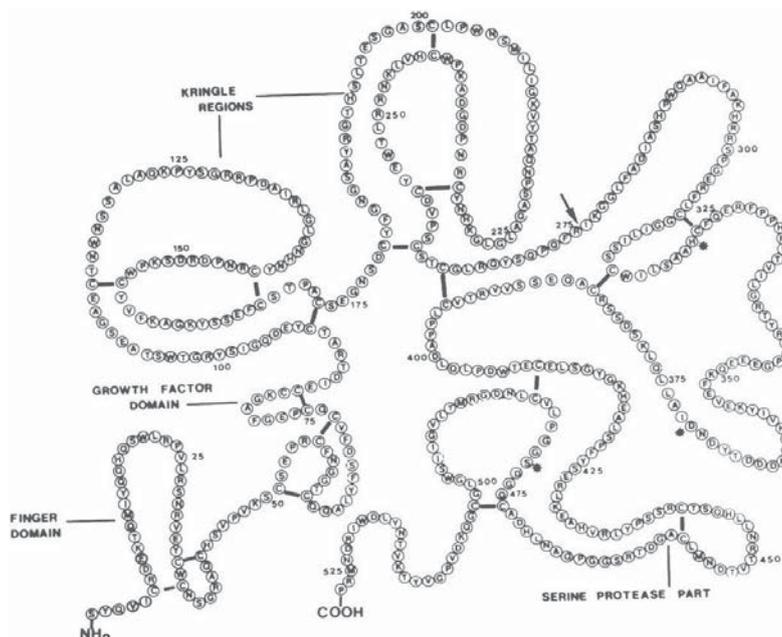


Figure 11. Schematic representation of the primary structure of tPA. The amino acids are represented by their single-letter symbols and black bars indicate disulphide bonds. The active site residues His322, Asp371 and Ser478 are marked by asterisks. The arrow indicates the cleavage site for the conversion of single-chain tPA to two-chain tPA (adopted from Collen & Linjen, 2009).

Unlike uPA, tPA has high affinity for fibrin, which is attributed to the presence of the finger domain and two kringle domains (Castellino & Ploplis, 2003; Politis, 1996; Stoppelli, 2000). Unlike uPA, plasminogen activation is enhanced by tPA in the presence of a plasmin substrate, e.g. fibrin, which is attributed to the binding of tPA to fibrin, resulting in a conformational change and a decrease in the Michaelis constant of tPA for plasminogen (Politis, 1996). The mechanism is mainly attributed to the exposure of COOH-terminal lysine and arginine residues, resulting in the tighter association of both tPA and plasminogen with a fibrin-formed blood clot (Castellino & Ploplis, 2003).

### **2.2.3 Plasmin system inhibitors**

The conversion of plasminogen to plasmin and proteolysis in the milk system by plasmin can be controlled by inhibition of the PA or plasmin by a plasminogen activator inhibitor (PAI) or a plasmin inhibitor (PI). Plasmin and PAs are serine proteases and can be controlled by serine protease inhibitors known as 'serpins' i.e.  $\alpha$ 2-antiplasmin (a PI) (Gils & Declerck, 2003).

#### ***Plasminogen activator inhibitors***

PIs directly inhibit plasmin activity whereas a PAI controls the activity of PAs. The speed of the interaction between activators and inhibitors plays an important role, e.g. fast-acting PAI rapidly inactivates PAs (Politis, 1996). PAI-1 and PAI-2 are the two major PAIs that have been identified and both have been found to form equimolar complexes (serine protease complexes – the reactive peptide bond of the inhibitor reacts with the active site of serine of the protease) with uPA and tPA (Andreasen, Georg, Lund, Riccio, & Stacey, 1990; Politis, 1996). PAI-1 inhibits tPA and uPA very rapidly, with high second order rate constants ( $10^7 \text{ M}^{-1}\text{s}^{-1}$  for uPA), and inhibits plasmin and trypsin much more slowly (second order rate constant of  $6.6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  for plasmin) (Gils & Declerck, 2003). PAI-1 controls cell-associated uPA in two ways: by suppressing its proteolytic activity and by reducing the amount of surface-bound uPA (Crippa, 2007).

### ***Plasmin inhibitors***

Christensen, Wiegers, Hermansen, and Sottrup-Jensen (1995b) identified seven different types of bovine PIs from bovine colostrum and milk, namely  $\alpha$ 2-antiplasmin, antithrombin III,  $\alpha$ 2-macroglobulin, C1-inhibitor, inter- $\alpha$ -trypsin inhibitor, plasma elastase inhibitor and bovine plasma trypsin inhibitor.

Christensen and Sottrup-Jensen (1992) purified and characterised bovine  $\alpha$ 2-antiplasmin and found it to be highly homologous to human  $\alpha$ 2-antiplasmin in amino acid composition and sequence, particularly sequences at the N terminus and the reactive site. The low molecular masses of bovine  $\alpha$ 2-antiplasmin and bovine PAI-1 (50 kDa) facilitate easy migration from blood to milk, making them a major PI and a major PAI in the control of the milk plasmin system in bovine milk (Precetti et al., 1997).  $\alpha$ 2-Antiplasmin is a specific PI and reacts rapidly to inactivate plasmin, whereas  $\alpha$ 2-macroglobulin, although present most prominently, is non-specific and reacts with all kinds of protease (Politis, 1996).

### ***Other inhibitors***

Kunitz and Northrop (1936) isolated a trypsin inhibitor from bovine pancreas that can inhibit all serine proteases; it was later known as bovine pancreatic trypsin inhibitor (BPTI) or aprotinin (a Kunitz<sup>2</sup>-type serine PI; relatively small; about 50–60 amino acids; ~ 6 kDa) (Ascenzi et al., 2003; Kunitz & Northrop, 1936). The PI aprotinin is the most widely used antifibrinolytic for perioperative bleeding (Swedberg & Harris, 2011). Aminocaproic acid ( $\epsilon$ -aminocaproic acid) and other lysine analogues bind to plasminogen's lysine-binding site and prevent attachment to fibrin, ultimately decreasing the conversion of plasminogen to plasmin (Swedberg & Harris, 2011).

Bovine milk PAs are inhibited by TLCK (tosyllysine chloromethyl ketone), PMSF (phenylmethylsulphonyl fluoride: binds specifically to the active site serine residue),

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<sup>2</sup> A Kunitz domain is the active domain of a protein that inhibits the function of protein-degrading enzymes.

TPCK (tosyl phenylalanyl chloromethyl ketone: the phenylalanine moiety is bound to the enzyme because of specificity for aromatic amino acid residues at the active site), PAI-1, PAI from erythrina seed and human plasma  $\alpha$ 1-antiplasmin; however, they are not inhibited by human plasma  $\alpha$ 1-antitrypsin (Lu & Nielsen, 1993c).

### **2.3 DISTRIBUTION OF THE PLASMIN SYSTEM IN MILK**

The presence of kringle domains in the plasminogen structure plays the most important role in the attachment or high affinity of plasminogen to fibrin, and bacterial and mammalian cell surfaces (Castellino & Ploplis, 2003; Suenson & Thorsen, 1981). Markus, DePasquale, and Wissler (1978) found one strong binding site ( $K = 0.009$  mM) and approximately five weaker sites ( $K = 5$  mM) on human plasminogen that bind with  $\epsilon$ -aminocaproic acid. Plasminogen and its activators are associated mainly with casein micelles and can be separated by certain treatments (de Rham & Andrews, 1982; Korycha-Dahl et al., 1983; Reimerdes & Klostermeyer, 1974; Richardson, 1983a), whereas inhibitors of plasmin and PAs are located in the serum fraction of milk (Deharveng & Nielsen, 1991; Korycha-Dahl et al., 1983; Reimerdes, Klostermeyer, & Sayk, 1976).

Similar to the blood plasminogen system, the presence of several kringle structures in bovine milk plasminogen could be the main reason for its association with casein micelles (Grufferty & Fox, 1988d; Politis et al., 1992). Several workers have reported the association of plasmin and plasminogen with the casein fraction of milk (plasmin–casein interactions) (de Rham & Andrews, 1982; Eigel et al., 1979; Fox, 1981b; Korycha-Dahl et al., 1983; Reimerdes & Klostermeyer, 1974; Richardson, 1983a; Snoeren & van Riel, 1979); the association can shift from casein to the whey fraction under specific conditions (Benfeldt et al., 1995; Fajardo-Lira & Nielsen, 1998). Benfeldt et al. (1995) confirmed the association of plasminogen with acid-precipitated casein, rennet-coagulated casein and casein micelles (Grufferty & Fox, 1988a; Richardson & Elston, 1984). According to Politis et al. (1992), most of the plasmin and plasminogen attaches to the casein fraction of milk through kringles, whereas the absence of a kringle structure in PAIs could be the main reason for their presence in the serum fraction of milk (Politis et al., 1992). Plasminogen also

utilises kringle domains (all but K3) for interaction with small molecules such as  $\text{Cl}^-$  and  $\omega$ -amino acids (negative or positive effector of plasminogen activation) (Urano et al., 1987a, 1987b). The association of plasmin with the milk fat globule membrane (MFGM) has also been reported from immunological experiments (Hofmann et al., 1979), which is suggested to be due to contamination of casein or casein association with the MFGM (Politis et al., 1992); this was also verified by the removal of plasminogen from MFGM along with casein (Benfeldt et al., 1995).

The association of plasminogen with casein is influenced by the presence of  $\epsilon$ -aminocaproic acid, lysine and its analogues, which eliminates lysine binding to casein and releases plasminogen from casein micelles (Baer et al., 1994; Benfeldt et al., 1995; Eigel et al., 1979; Korycha-Dahl et al., 1983; Richardson, 1983a); higher concentrations ( $\sim 80$  mM) inhibit plasmin activity and incubation with 50 mM  $\epsilon$ -aminocaproic acid was found to enhance plasminogen activation (Bastian & Brown, 1996; Nielsen, 2003). The effect of  $\epsilon$ -aminocaproic acid and lysine derivatives on plasminogen association suggests the importance of the lysine-binding site present on kringle domains in the attachment of plasminogen to the casein micelle; later, it was suggested that the amount of plasminogen bound to casein depends on the type of casein, the lysine-binding sites and electrostatic forces and it was also suggested that the role of electrostatic forces becomes more important for immobilised casein (Baer et al., 1994). The association of midi-plasmin(ogen) with the casein micelle is suggested to be weaker than that of plasmin(ogen) and is observed especially in whey and the serum fraction of milk, which is suggested to be due to less lysine-binding capability [only two kringles (K4 and K5) in midi-plasmin(ogen) compared with five in plasmin(ogen)] (Benfeldt et al., 1995).

The effects of different dairy processing parameters such as pH, temperature and ionic strength on the release of plasmin from the casein micelles in milk have been studied. Most plasmin is removed from the casein micelles below pH 4.6 (Grufferty & Fox, 1988a; Richardson & Elston, 1984). According to Baer et al. (1994), the binding of plasminogen to casein is at a maximum at pH 4.6 (the pI of casein), because of the very high charge difference between plasminogen (the pI of human plasminogen is between 6.2 and 6.6) and casein. A slight decrease in binding with

an increase in pH towards the pI of plasminogen and a complete loss of binding below pH 4.0 were observed. This indicates the role of electrostatic forces besides lysine-binding sites in the binding of plasminogen to casein (Baer et al., 1994).

According to Richardson and Elston (1984), during cheese making, acid precipitation of the casein micelle results in a loss of plasmin from the micelle because of the drastic disruption of the casein micelles, compared with rennet coagulation (sweet whey generation), which has less loss of plasmin activity from the casein micelle because the micelles are less disrupted. This is also supported by later reports showing higher plasmin activity in acid whey than in sweet (rennet) whey (Cruden & Kelly, 2003; Hayes & Nielsen, 2000).

The addition of 1 M NaCl results in complete loss of plasmin activity from the casein micelle (Grufferty & Fox, 1988a). According to Grufferty and Fox (1988c), storage temperature has little or no effect on the interaction between plasmin and casein, which conflicts with the results of Reimerdes and Herlitz (1979), who observed an increase in plasmin-induced hydrolysis in milk stored at low temperature and suggested that the dissociation of  $\beta$ -casein (forms monomers) and plasmin from casein micelles into the serum enhances the formation of  $\gamma$ -casein (Reimerdes & Herlitz, 1979). The growth of psychrotrophic bacteria and the production of protease are also found to release plasmin and plasminogen from the casein micelle into the whey fraction (Fajardo-Lira & Nielsen, 1998).

uPA and tPA have been shown to be produced and present in bovine mammary tissue (Heegaard et al., 1994b) and are also associated with the casein micelles in milk (Heegaard et al., 1994a; Lu & Nielsen, 1993c; Politis et al., 1991). Lu and Nielsen (1993) reported the presence of five different PAs, presumably uPA in the casein fraction, in contrast to Heegaard et al. (1994a), who reported that tPA is the main PA that is associated with the casein micelles. This was contradicted by Zachos et al. (1992), who suggested the association of tPA with somatic cells; however, this was later shown to be due to the presence of casein micelles entrapped in somatic cells (White et al., 1995). Work by White et al. (1995) clearly suggests that tPA is associated with casein micelles and that uPA is associated with somatic cells.

tPA is the main PA that is associated with casein micelles (Heegaard et al., 1994a; White et al., 1995); it cannot be released by  $\epsilon$ -aminocaproic acid, whereas it is released in a dose-dependent manner by treatment with NaCl. It was suggested that the association of tPA with casein micelles is due to electrostatic forces, which is different from the role of lysine-binding sites in the association of plasminogen with casein micelles (Heegaard et al., 1994a). uPA is the predominant form of PA that is associated with milk somatic cells (White et al., 1995), which can be attributed to the presence of the uPA receptor in milk somatic cells. Heegaard et al. (1994a) found a high concentration of uPA receptor in milk somatic cells isolated from healthy quarters as well as from mastitic quarters (Politis, 1996).

The epidermal-growth-factor-like domain and the kringle in uPA make up the amino-terminal fragment, which mediates the binding of uPA to its receptor, which is present in numerous cells, especially the somatic cells in milk. tPA consists of two kringle structures that play an important role in its binding to fibrin (Politis, 1996) and possibly to casein micelles, similar to plasminogen. Unlike PAs, PIs and PAIs are localised in the milk serum (Deharveng & Nielsen, 1991).

Some researchers have found that both human caseins and bovine caseins [especially  $\alpha_s$ -caseins ( $\alpha$ -casein >  $\kappa$ -casein >  $\beta$ -casein)] enhance the rate of plasminogen activation by both tPA and uPA; this is attributed to extensive binding of plasminogen and PAs to caseins (Heegaard et al., 1994a; Markus et al., 1993; Politis et al., 1995) and it is also suggested that the caseins play a role as an immobilisation matrix for plasminogen activation in milk (Baer et al., 1994; Politis et al., 1995), especially multimers of  $\kappa$ -casein and dimers of  $\alpha_{s2}$ -casein (Heegaard et al., 1994a). Although casein and fibrin have several analogies, uPA activity on human Lys-plasminogen is not enhanced by fibrin, as is tPA activity (Bastian & Brown, 1996).

## **2.4 MILK PROTEINS**

Bovine milk contains approximately 3.0–3.5% proteins. These milk proteins are divided into two broad classes, based on pH-dependent precipitation. One fraction, consisting of 80% of the total protein, is precipitated at pH 4.6 (isoelectric pH) at

30°C and is known as casein; the other fraction (20% of the total protein) is soluble under these conditions and is known as whey protein or serum proteins (Fox & McSweeney, 1998).

Table 1. Distribution of milk proteins

Protein fraction	%
<b>Caseins</b>	2.4–2.8
β-Casein	0.9–1.1
α <sub>s1</sub> -Casein	1.1–1.5
α <sub>s2</sub> -Casein	0.3–0.4
κ-Casein	0.3–0.4
<b>Whey proteins</b>	0.5–0.7
β-Lactoglobulin	0.2–0.4
α-Lactalbumin	0.1–0.15
Immunoglobulins	0.06–0.1
Serum albumin	0.01–0.04
Lactoferrin	0.01

#### 2.4.1 Caseins

The caseins in bovine milk are present in the form of a colloidal dispersion known as the casein micelle (Schmidt, 1982). The structure and the properties of the casein micelle have been extensively studied and reviewed (Creamer, 1991b; Dalgleish, 1998, 2007, 2010; de Kruif, 1999; de Kruif & Holt, 2003; de Kruif, Huppertz, Urban, & Petukhov, 2012; Fox & Brodtkorb, 2008; Holt, 1992; Horne, 2006, 2008; Tuinier & de Kruif, 2002). The casein micelles have a diameter of 50–500 nm (average ~ 150 nm) and a molecular weight ranging from 10<sup>6</sup> to 10<sup>9</sup> Da (average ~ 10<sup>8</sup> Da), comprise approximately 10<sup>4</sup> casein molecules and contain inorganic matter, mainly calcium phosphate, about 8 g/100 g casein (Walstra, Wouters, & Guerts, 2005). The casein micelle is composed of the main four caseins: α<sub>s1</sub>-casein, α<sub>s2</sub>-casein, β-casein and κ-casein (molar ratio 4:1:4:1 respectively) (de Kruif & Holt, 2003; Swaisgood, 2003). These caseins have differences in post-translational processing such as phosphorylation, glycosylation and limited proteolysis (Swaisgood, 2003). None of the caseins appears to have extensive amounts of secondary structure; neither do they possess well-defined permanent tertiary structures. This could mainly be due

to high levels of proline residues ( $\alpha_{s1}$ -casein – 17 proline residues,  $\alpha_{s2}$ -casein- 10 ,  $\beta$ -casein- 35 and  $\kappa$ -casein-20) and their fairly uniform distribution. They have been described as ‘rheomorphic’ or ‘natively disordered’, implying that they may adapt their structures to suit the conditions (Dalglish, 2010). Among the caseins,  $\alpha_{s1}$ -casein has the highest charge, whereas  $\alpha_{s2}$ -casein contains two cysteine residues, forming –S–S– bridges, and the highest phosphate content. Most  $\kappa$ -casein molecules are glycosylated to various extents (Swaisgood, 2003).  $\beta$ -Casein is the most hydrophobic casein and both  $\beta$ -casein and  $\kappa$ -casein have two distinct hydrophilic charged blocks and a hydrophobic block (Horne, 2002). After storage for some hours at low temperature,  $\beta$ -casein concentration in serum phase reversibly increases (Creamer, Berry, & Mills, 1977). The enzymatic degradation of  $\beta$ -casein by plasmin (alkaline milk protease) leads to the production of  $\gamma$ -caseins and proteose peptones (Ismail & Nielsen, 2010); in contrast,  $\kappa$ -casein is resistant to plasmin but is readily hydrolysed by rennet (chymosin) and forms para- $\kappa$ -casein and glycomacropeptide (Lawrence & Creamer, 1969).  $\kappa$ -Casein has two cysteine residues and interacts with whey proteins through disulphide bonds upon heat treatment (Donato & Guyomarc’h, 2009). Casein micelles consist of caseins held together by hydrophobic interactions and by the bridging of calcium phosphate nanoclusters bound to serine phosphate residues of the  $\alpha_{s1}$ -,  $\alpha_{s2}$ - and  $\beta$ -caseins (de Kruif & Holt, 2003).  $\kappa$ -Casein is thought to limit the process of self-association, leading to stabilisation of the native casein micelle (de Kruif et al., 2012); it is considered to be present as a brush of grafted polymer on the surface, protruding into the solvent to provide the steric stabilisation of the micelle (de Kruif & Zhulina, 1996). Casein micelles are very stable to the principal processes to which milk is subjected: high temperatures, compaction and commercial homogenisation (Fox, 2003).

Table 2. Properties of some milk proteins (Fox & McSweeney, 1998)

Properties	Caseins				$\beta$ -Lg	$\alpha$ -La
	$\alpha_{s1}$	$\alpha_{s2}$	$\beta$ -	$\kappa$ -		
MW	23 612	25 228	23 980	19 005	18 362	14 174
Residues	199	207	209	169	162	123
Conc in milk (g/L)	12–15	3–4	9–11	2–4	3.0	0.7
Phosphate residues	8–9	10–13	4–5	1–2	0	0
½ Cystine	0	2	0	2	5	8
Sugars	0	0	0	Yes	0	0
Prolyl residue per molecule	17	10	35	20	8	2
A <sub>280</sub> , 1% 1 cm	10.1	11.1	4.6	9.6	9.4	20.1
Secondary structure	Low	Low	Low	Low	High	High
H $\phi_{ave}$	4.89	4.64	5.58	5.12	5.03	4.68
pI	4.96	5.27	5.20	5.54	5.2	4.2–4.5
Partial specific volume (ml/g)	0.728	0.720	0.741	0.734	0.751	0.735

## 2.4.2 Whey proteins

Whey proteins are soluble at pH 4.6, soluble after rennet coagulation, separated from the casein micelle by microfiltration and gel filtration and not sedimented by ultracentrifugation (with or without added Ca<sup>2+</sup>) (Fox, 2003). Whey proteins are stable against renneting and are relatively heat labile, being completely denatured by heating at 90°C for 10 min (Fox & McSweeney, 1998). Whey proteins have two well-defined groups; based on fractionation by saturated MgSO<sub>4</sub>, the precipitate was referred to as lactoglobulin and the soluble protein was referred to as lactalbumin (Fox & McSweeney, 1998). The lactalbumin fraction of bovine milk contains three main proteins, i.e.  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin and bovine serum albumin, representing approximately 50, 20 and 10% of the total whey protein respectively, and trace amounts of several other proteins, notably lactotransferrin, serotransferrin and several enzymes. The lactoglobulin fraction consists mainly of immunoglobulins (Fox & McSweeney, 1998).

## 2.5 PLASMIN AND PROTEOLYSIS OF MILK PROTEINS

The plasmin system is indigenously present in milk and causes hydrolysis of the milk proteins. The association of plasmin with casein (de Rham & Andrews, 1982; Korycha-Dahl et al., 1983; Reimerdes & Klostermeyer, 1974; Richardson, 1983a) and the role of casein in plasminogen activation have been studied by several

researchers (Baer et al., 1994; Heegaard et al., 1994a; Politis et al., 1995). Plasmin specifically hydrolyses Lys–X and Arg–X bonds, similar to trypsin (serine protease) but with more selectivity (Bastian & Brown, 1996), and its regulation in the manufacture of dairy products is critically important. Plasmin hydrolyses primarily  $\beta$ -casein and  $\alpha_{s2}$ -casein (Andrews & Alichanidis, 1983; Eigel, 1977b; Le Bars & Gripon, 1989; Richardson, 1983a; Snoeren & van Riel, 1979; Visser, Noorman, Slangen, & Rollema, 1989a; Visser, Slangen, Alting, & Vreeman, 1989b), whereas its hydrolysis of  $\alpha_{s1}$ -casein is slower (Andrews, 1983; Coker, Creamer, Burr, & Hill, 1999; de Rham & Andrews, 1982; Eigel, 1977a; Le Bars & Gripon, 1993; McSweeney, Olson, Fox, Healy, & Hojrup, 1993) and  $\kappa$ -casein is more resistant to hydrolysis (Chen & Ledford, 1971; Eigel, 1977a; Kaminogawa et al., 1972). The whey proteins  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin are little or not affected by plasmin activity (Bastian & Brown, 1996; Caessens, Daamen, Gruppen, Visser, & Voragen, 1999a; Caessens, Visser, Gruppen, & Voragen, 1999b). Caseins are unstructured and have more chain flexibility than globular whey proteins, which make them more susceptible to proteolysis.

### 2.5.1 Plasmin action on $\beta$ -casein

$\beta$ -Casein consists of 209 residues and has a molecular weight of 23,980 Da (Ribadeau-Dumas, Brignon, Grosclaude, & Mercier, 1972). Plasmin hydrolyses  $\beta$ -casein rapidly and produces the  $\gamma$ -casein and proteose peptone fractions (Andrews, 1978a, 1978b; Eigel, 1977b; Eigel & Keenan, 1979; Kaminogawa et al., 1972; Snoeren & van Riel, 1979).  $\beta$ -Casein bands disappear with a concomitant appearance  $\gamma_1$ -,  $\gamma_2$ - and  $\gamma_3$ -casein bands on AU-PAGE (Snoeren & van Riel, 1979).  $\gamma$ -Caseins are C-terminal hydrophobic domains derived from  $\beta$ -casein after plasmin hydrolysis and can be extracted in organic solvents. A close relationship between several minor fractions ( $\gamma_1$ -,  $\gamma_2$ -,  $\gamma_3$ -caseins) and  $\beta$ -casein was observed through amino acid analysis, molecular weight determination, peptide mapping and end-group analysis (Gordon et al., 1972; Groves, Gordon, Kalan, & Jones, 1972). These minor fractions were found to be identical to the 29–209, 106–209 and 108–209 residues of  $\beta$ -casein respectively (Eigel, 1977b; Ribadeau-Dumas et al., 1972). Andrews (1978a, 1978b) demonstrated that the N-terminal portion of  $\beta$ -casein that

was generated after plasmin hydrolysis corresponded to proteose peptone fractions 5 and 8 (PP5 and PP8); these fractions are present in milk in low concentration. PP5 consists of two mixed peptides, i.e. 1–105 and 1–107 (Andrews, 1978a). PP8fast is the 1–28 N-terminal sequence residues of  $\beta$ -casein (Andrews, 1978b) and the  $\gamma$ -caseins represent the matching C-terminal sequence residues of  $\beta$ -casein (Andrews, 1978a, 1978b). Eigel and Keenan (1979) suggested the formation of PP8slow (molecular weight 8800 Da), consisting of the  $\beta$ -casein fragments 29–105 and 29–107, during the hydrolysis of  $\gamma_1$ -casein by plasmin to form  $\gamma_2$ -casein and  $\gamma_3$ -casein respectively. The proteose peptones (polar domains) are highly soluble and heat stable whereas the  $\gamma$ -caseins (hydrophobic domains) are hydrophobic and can be extracted in organic solvents (Reimerdes & Herlitz, 1979; Swaisgood, 2003).

Visser et al. (1989a) used a membrane reactor for the plasmin-induced hydrolysis of  $\beta$ -casein and identified the formation of 16 peptide fragments. The N-terminal half of  $\beta$ -casein was found to be more sensitive to plasmin hydrolysis than the rest of the molecule. This could be because hydrophobic interactions at the C-terminal part of  $\beta$ -casein cause it to aggregate at higher temperature.

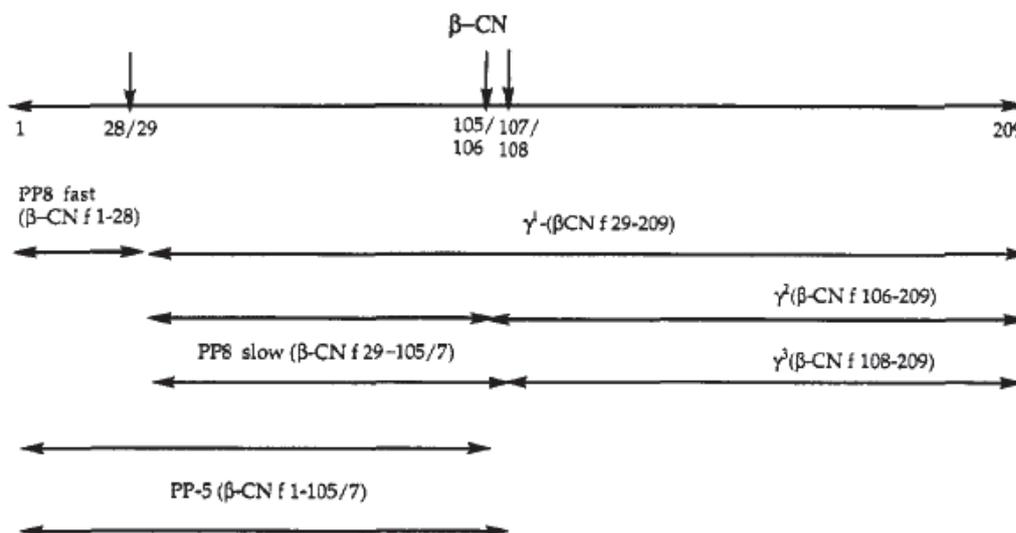


Figure 12. Principal products produced from  $\beta$ -casein by plasmin (Fox & McSweeney, 1998).

### 2.5.2 Plasmin action on $\alpha_{s1}$ -casein

Plasmin hydrolyses  $\alpha_{s1}$ -casein more slowly than  $\beta$ -casein. Eigel (1977a) first studied the hydrolysis of  $\alpha_{s1}$ -casein and described the generation of hydrolysis products with higher electrophoretic mobility than  $\alpha_{s1}$ -casein. Aimutis and Eigel (1982) also found similar products with molecular weights of 5500 and 6000 Da, i.e. analogues of the  $\lambda$ -casein fractions (Aimutis & Eigel, 1982; Eigel, 1977a).

According to Le Bars and Gripon (1993), plasmin hydrolyses seven Lys–X and four Arg–X peptide bonds in  $\alpha_{s1}$ -casein, resulting in the formation of 15 peptides. McSweeney et al. (1993) found principal plasmin cleavage sites at Arg22–Phe23, Arg90–Tyr91, Lys102–Lys103, Lys103–Tyr104, Lys105–Val106, Lys124–Glu125 and Arg151–Gln152 and 11 other sites and suggested that plasmin hydrolyses  $\alpha_{s1}$ -casein initially towards the centre of the molecule and that 12 Lys–X and 5 Arg–X bonds are sensitive to plasmin.

Coker et al. (1999) studied the plasmin hydrolysis rates of different variants of  $\alpha_{s1}$ -casein (A, B and C variants); they found the same rates for all variants but different mobilities of the generated peptides on SDS-PAGE. The  $\alpha_{s1}$ -casein A variant peptides had higher mobilities than the most mobile peptides released from the  $\alpha_{s1}$ -casein B and C variants and this difference was attributed to the difference in peptide size and/or charge (Coker et al., 1999). According to McSweeney et al. (1993), Arg22–Phe23 is the primary site and is hydrolysed rapidly by plasmin; this was not supported by Coker et al. (1999), who suggested that Arg22–Phe23 is not the primary hydrolysis site because of the similar rates of loss of  $\alpha_{s1}$ -casein in all three variants (A, B and C).

### 2.5.3 Plasmin action on $\alpha_{s2}$ -casein

Plasmin hydrolyses  $\alpha_{s2}$ -casein at the same rate as  $\beta$ -casein and generates several peptides with high electrophoretic mobility (Snoeren & van Riel, 1979). Eight peptide bonds in  $\alpha_{s2}$ -casein were found to be hydrolysed by plasmin, seven Lys–X bonds and one Arg–X bond (Le Bars & Gripon, 1989; Visser et al., 1989b). Le Bars and Gripon (1989) found more plasmin-sensitive bonds in  $\alpha_{s2}$ -casein than in  $\beta$ -

casein and attributed this to the higher number of lysyl residues (24 versus 11) in  $\alpha_{s2}$ -casein. They found five sensitive linkages, 149–150, 24–25, 197–198, 150–151 and 21–22, with cleavage intensities of 6.8, 5.7, 5.6, 5.6 and 4.5%, and three linkages, 114–115, 181–182 and 188–189 with low cleavage intensities of 1.7, 1.4 and 2.3% respectively (Le Bars & Gripon, 1989). Three short peptides (198–207, 182–207 and 189–207) were generated from the C-terminal side of  $\alpha_{s2}$ -casein, were found to be very hydrophobic (1.76, 1.54 and 1.79 kcal/residue) and, based on similarity in hydrophobicity to the bitter peptides of  $\beta$ -casein (Visser, Slangen, Hup, & Stadhouders, 1983), were considered to be bitter peptides (Le Bars & Gripon, 1989). Most of the released peptides were soluble at pH 4.6. Harwalkar and Vreeman (1978) found no  $\gamma$ -casein formation in stored UHT-treated concentrated milk but  $\alpha_{s2}$ -casein disappeared, with the appearance of a protein with an electrophoretic mobility of  $-0.12$ ; they suggested that  $\alpha_{s2}$ -casein is the preferred substrate compared with  $\beta$ -casein in the hydrolysis of concentrated milk by plasmin (Harwalkar & Vreeman, 1978; Snoeren & van Riel, 1979).

#### **2.5.4 Plasmin action on $\kappa$ -casein**

Eigel (1977b) incubated  $\kappa$ -casein with plasmin for various times, did not find any changes in  $\kappa$ -casein's electrophoretic patterns and concluded that  $\kappa$ -casein was resistant to hydrolysis by plasmin, which was in agreement with other researchers (Chen & Ledford, 1971; Eigel, 1977a). According to Doi, Kawaguchi, Ibuki, and Kanamori (1979), the attachment of carbohydrate moieties to  $\kappa$ -casein prevents its hydrolysis by plasmin; in the absence of carbohydrate, it was found to be susceptible to hydrolysis by plasmin. The resistance of  $\kappa$ -casein to plasmin was not altered even after removal of sialic acid from  $\kappa$ -casein by treatment with neuraminidase (Eigel, 1977a). The specificity for the hydrolysis by plasmin of  $\kappa$ -casein has not been determined (Grufferty & Fox, 1988d). However, Andrews and Alichanidis (1983) observed that 4% peptides (out of 52% total hydrolysed peptides) were generated from the plasmin hydrolysis of  $\kappa$ -casein, which could have been due to differences in the conditions and concentrations of the enzymes and substrates used (Grufferty & Fox, 1988d).

The hydrolysis of  $\kappa$ -casein by plasmin was observed during involution of the mammary gland by immunoblot analysis (Aslam & Hurley, 1997). The para- $\kappa$ -casein generated after the rennet hydrolysis of  $\kappa$ -casein was found to be susceptible to plasmin hydrolysis (Trujillo, Guamis, & Carretero, 1998).

### **2.5.5 Plasmin action on whey proteins**

Unlike the caseins, the whey proteins  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin are resistant to hydrolysis by the milk protease plasmin, because of their rigid globular conformations (Chen & Ledford, 1971; Yamauchi & Kaminogawa, 1972). Aslam and Hurley (1997) did not observe any proteolytic cleavage of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin, but did observe proteolytic cleavage of lactoferrin, in mammary secretions during involution. Caessens et al. (1999a, 1999b) observed hydrolysis of  $\beta$ -lactoglobulin by plasmin and found that the hydrolysates of  $\beta$ -lactoglobulin that were generated exhibited good functional properties.

## **2.6 FACTORS AFFECTING THE ACTIVITY OF THE PLASMIN SYSTEM IN MILK**

The activity of plasmin in the milk system is affected by many factors, e.g. stage of lactation, mastitis, age of the cow and breed of the cow, because of altered transport of the enzyme from milk or different degrees of plasminogen activation (Kelly & McSweeney, 2003). Different processing parameters, such as heat treatment, storage temperature and the cheese manufacturing process, also affect the activity of plasmin in different milk systems.

### **2.6.1 Stage of lactation**

The plasmin and plasminogen concentrations and/or the derived activities are affected by the stage of lactation. In the advanced stages of lactation, the concentrations of plasmin and plasminogen (Bastian, Brown, & Ernstrom, 1991a; Nicholas, Auld, Molan, Stelwagen, & Prosser, 2002; Politis, Lachance, Block, & Turner, 1989a; Richardson, 1983b; Schaar, 1985) and the PA activity (Baldi et al., 1996; Gilmore, White, Zavizion, & Politis, 1995) increase, mainly because of increased permeability of the blood vessels in the mammary glands (as the plasmin

system is derived from the blood in milk) (Korycha-Dahl et al., 1983; Richardson, 1983b). According to Politis et al. (1989a), plasminogen activation increases during late lactation, which is in contrast to Richardson (1983b), who suggested that the increase in plasmin activity is due mainly to the entry of more plasmin from the blood to the milk rather than an increase in plasminogen activation; this is supported by the fact that loosening of mammary tight junctions occurs during advancing lactation (Stelwagen et al., 1994). Nicholas et al. (2002) supported the results of Richardson (1983b) and showed less activation of plasminogen during mid and late lactation than in early lactation.

Bastian et al. (1991a) suggested that greater activation of plasminogen in late lactation also agrees with the results of Politis et al. (1989a), whereas the flow of the enzyme from the blood into the milk increases in early lactation and remains constant at the end of lactation. Baldi et al. (1996) observed increases in plasmin, plasminogen and PA and a reduction in the plasminogen:plasmin ratio with advancing lactation. The increase in the level of plasmin with advancing lactation is due to the increase in the level of plasminogen and its accelerated conversion to plasmin, which is positively correlated with the somatic cell count (SCC) (Baldi et al., 1996; Fox, 2002).

### **2.6.2 Mammary pathology: mastitis**

The proteolytic activity of plasmin increases in mastitic milk (Politis et al., 1989a; Politis, Ng Kwai Hang, & Giroux, 1989b; Saeman, Verdi, Galton, & Barbano, 1988; Schaar & Funke, 1986). Increases in the plasmin and plasminogen concentrations and activities were observed with an increase in SCC (Gilmore et al., 1995; Politis et al., 1989a; Zachos et al., 1992). Politis et al. (1989a) observed increases in the concentration from 0.18 to 0.37 mg/L for plasmin and from 0.85 to 1.48 mg/L for plasminogen with an increase in the SCC from 250,000 to > 1 million cells/mL; however, the ratio of plasminogen to plasmin was decreased, indicating that the increase in plasmin was due mainly to the increase in the transfer of plasminogen from the blood to the milk rather than to accelerated conversion of plasminogen to plasmin. Such a mechanism is different from that in late lactation, in which the

predominant event is accelerated conversion of plasminogen to plasmin (de Rham & Andrews, 1982; Gilmore et al., 1995; Schaar & Funke, 1986).

According to Verdi and Barbano (1991), somatic cells are the main source of PA in milk, suggesting that milk with a high SCC will have high plasmin activity, which is in agreement with the results of Politis et al. (1989b), whereas leukocytes, milk coagulants and extracellular bacterial enzymes cannot activate plasminogen. Zachos et al. (1992) measured the PA activity in somatic cells and found an eightfold higher activity than in healthy quarters; Heegaard et al. (1994a) observed a 10–20-fold increase in the uPA and tPA concentrations in milk from mastitic quarters. Gilmore et al. (1995) measured the PA activity in the casein fraction of milk and found that it was 8.5- and 3.2-fold higher in high SCC milk than in low and medium SCC milk respectively; they suggested that the PA level increases when the SCC is  $> 7.5 \times 10^5$  cells/mL. The higher concentration of PA in mastitic milk favours the activation of plasminogen. Politis et al. (1991) found that PA was produced from bovine milk macrophages and blood monocytes. According to Heegaard et al. (1994a), uPA is bound to the uPA receptor by mammary neutrophils, which are up-regulated during intramammary infection. Schaar and Funke (1986) observed dissociation of plasminogen and plasmin from the casein micelles in mastitic milk and found a greater degree of activation of plasminogen associated with casein than of soluble plasminogen in mastitic milk.

### **2.6.3 Effect of age and breed of the cow, season and milking frequency**

The activity of plasmin differs between breeds of cattle, with milk from Swedish Red and White having the highest activity, followed by Swedish Friesian milk, and Jersey milk having the lowest activity. Schaar (1985), in agreement with Richardson (1983b), observed higher plasmin activity in Holstein–Friesian milk (0.27–0.53 mL/L) than in Jersey milk (0.15–0.37 mg/L) (Richardson, 1983b). However, Bastian et al. (1991a) found no significant difference between milks from Holstein and Jersey cows after adjustment of the casein:substrate ratio and suggested that differences in plasmin activity between breeds are due to differences in the casein content of

the milk, which interferes with the substrate during activity measurement (Bastian et al., 1991a; Schaar, 1985).

Plasmin activity also increases in milk from older cows (Bastian et al., 1991a; Politis et al., 1989b; Schaar, 1985). According to Saeman et al. (1988), even after curing mastitis, plasmin activity does not return to its pre-infection level, which could be one reason for the higher plasmin activity in milk from older cows than in milk from young cows. A decrease in milking frequency also causes increases in the activities of plasminogen, plasmin and PA in milk, with an accelerated conversion of plasminogen to plasmin (Kelly, Reid, Joyce, Meaney & Foley, 1998; Stelwagen et al., 1994). Milk samples obtained in autumn and winter have higher plasmin activity (Bastian et al., 1991a; Politis et al., 1989b); this is opposite to the observation of Nicholas et al. (2002), who found the highest plasmin + plasminogen activity in spring followed by summer. Such differences in the results could have been due to different conditions between the northern hemisphere (Bastian et al., 1991a; Politis et al., 1989b) and New Zealand (Nicholas et al., 2002).

#### **2.6.4 Heat treatment**

Plasmin and plasminogen are little affected by pasteurisation conditions and partially survive UHT processing (Driessen, 1983; Driessen & van der Waals, 1978; Dulley, 1972; Metwalli et al., 1998). Reported D values for plasmin are 35.7 min (Driessen & van der Waals, 1978) and 12.4 min (Alichanidis et al., 1986) at 72.5°C and 7 s (Driessen & van der Waals, 1978) and 10 s (Alichanidis et al., 1986) at 142.5°C. PAs are also very heat stable and have D values of 109 min at 70°C and 32 s at 140°C (Lu & Nielsen, 1993b), with uPa being more thermally stable than tPA (Prado et al., 2007). The inactivations of plasmin, plasminogen and PAs follow first order kinetics (Alichanidis et al., 1986; Borda, Rotaru, Costin, & Hendrickx, 2003; Kennedy & Kelly, 1997; Lu & Nielsen, 1993b).

According to Metwalli et al. (1998), the high heat stability of plasmin in milk products appears to be due not to a high conformational stability but to protection by casein towards irreversible inactivation of the unfolded enzyme. Plasmin was found to be more stable in skim milk than in buffer alone because of substrate

protection of plasmin by casein; this protection was found to decrease or to be lost at higher temperatures (Alichanidis et al., 1986). The heat stability of plasmin increases in the presence of casein (because of substrate protection), whereas heat inactivation of the plasmin system is enhanced in the presence of  $\beta$ -lactoglobulin and especially free –SH groups by thiol–disulphide interactions (Alichanidis et al., 1986; Grufferty & Fox, 1988c; Metwalli et al., 1998; Rollema & Poll, 1986; Saint Denis, Humbert, & Gaillard, 2001). Higher plasmin inactivation rates were observed in non-micellar casein solutions than in micellar casein solutions by Grufferty and Fox (1988b) and suggested that the micellar structure protects the enzyme against heat-induced denaturation. Further, the role of lysine-binding sites was also studied by adding L-lysine and  $\epsilon$ -aminocaproic acid; lysine-binding sites were found to play an important role in the binding of plasmin to casein and thus the increase in heat stability (Grufferty & Fox, 1988c).

The addition of  $\beta$ -lactoglobulin greatly accelerates the heat inactivation of plasmin, whereas  $\alpha$ -lactalbumin has no significant effect and a mixture of both  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin has an intermediate effect. Under reduced conditions, the inhibitory effect of  $\beta$ -lactoglobulin was also reduced. Ovalbumin and bovine serum albumin at a concentration of 10 mg/mL had similar inhibitory effects on plasmin to  $\beta$ -lactoglobulin (Alichanidis et al., 1986).  $\beta$ -Lactoglobulin contains two disulphide bonds and one –SH group per molecule, whereas  $\alpha$ -lactalbumin contains four disulphide bonds but no free –SH groups per molecule; this indicates the role of the free –SH group in the heat inactivation of plasmin. This was further supported by a decrease in or a loss of plasmin inactivation ability upon the carboxymethylation of the –SH group of  $\beta$ -lactoglobulin (Alichanidis et al., 1986) and upon the addition of potassium iodate ( $KIO_3$ ) to milk before UHT treatment (Kelly & Foley, 1997). Ovalbumin and bovine serum albumin also have –SH groups and were found to have similar inhibitory effects to  $\beta$ -lactoglobulin (Alichanidis et al., 1986). Small amounts of L-cysteine addition also increase the rate of heat inactivation of plasmin and plasminogen (Lu et al., 2009; Rollema & Poll, 1986). Heating of milk at higher temperature (above 70°C) denatures  $\beta$ -lactoglobulin and exposes the free –SH group (previously buried inside the native protein). Heat

treatment of plasminogen in the presence of a free –SH group results in an interchain disulphide-bonded complex and irreversible denaturation or unfolding (Alichanidis et al., 1986; Enright & Kelly, 1999; Metwalli et al., 1998; Rollema & Poll, 1986). Lu et al. (2009) observed a pronounced drop in plasminogen-derived plasmin activity compared with plasmin activity and indicated that plasminogen is more sensitive to free sulphhydryl groups than plasmin. The disulphide interchange reaction is faster at alkaline pH and Dulley (1972) observed rapid inactivation at higher pH.

Saint Denis et al. (2001) found a rapid decrease in the rate of plasmin inactivation during long heat treatment and attributed this to the disappearance of available  $\beta$ -lactoglobulin for S–S linking. The activation energies ( $E_a$ ) for the heat denaturation of plasmin, plasminogen and PAs were 29, 35 and 24 kJ/mol respectively in the temperature range 95–140°C and 244, 230 and 241 kJ/mol respectively in the temperature range 70–90°C (Saint Denis et al., 2001).

Plasmin system inhibitors are heat labile and are inactivated at low heat treatment. Prado et al. (2006) confirmed that there was almost complete inactivation of PAI on pasteurisation (75°C for 15 s) whereas PI retained 64% of its activity, which could be one of the reasons for the increase in plasmin activity and plasminogen activation on the pasteurisation of milk (Borda, Van Loey, Smout, & Hendrickx, 2004; de Rham & Andrews, 1982; Prado et al., 2006; Richardson, 1983a). In addition, the denaturation temperature range for bovine plasminogen is between 50.1 and 61.6°C (Burbrink & Hayes, 2006), meaning that plasminogen denatures and unfolds (unfolding of the kringle structure) at pasteurisation or similar temperatures and becomes more accessible to PA (Burbrink & Hayes, 2006; Lu et al., 2009). Thus, the inactivation of PAI and the denaturation of plasminogen play a role in the increased plasmin activity in pasteurised milk (Burbrink & Hayes, 2006). Restoration of plasmin activity was observed during storage and was attributed to the unfolding of plasminogen to PA attack and residual plasminogen and PA from the heat treatment (Lu et al., 2009). The results from two-dimensional electrophoresis confirmed the polymerisation of plasminogen into dimers, trimers and polymers

through disulphide interchange upon heating in the presence of  $\beta$ -lactoglobulin (Ismail & Nielsen, 2010).

### **2.6.5 Storage temperature**

The optimum temperature for plasmin activity and plasminogen activation is 37°C, but plasmin remains active during cold storage (Bastian & Brown, 1996). Any milk product with a storage temperature close to the optimum will have high plasmin activity, e.g. UHT milk, skim milk powder etc. Plasmin loses its activity upon prolonged storage because of autolysis, especially if partially unfolded by heat treatment (Crudden, Fox, & Kelly, 2005b; Huppertz, Fox, & Kelly, 2004). The presence of residual plasminogen and its activator can lead to the activation of plasminogen into plasmin during storage.

The effect of the storage temperature on the structure of the casein micelles in milk can also affect the hydrolysis of casein by plasmin. At lower storage temperatures,  $\beta$ -casein has a monomer form and becomes more soluble. In addition, according to Holt (1992), some  $\beta$ -casein is also bound to  $\kappa$ -casein and is involved in internal cohesion of the casein micelles; the preheating of milk releases a significant amount of  $\kappa$ -casein into the serum. Such changes allow plasmin to hydrolyse  $\beta$ -casein more easily, resulting in elevated hydrolysis during the refrigerated storage of milk (Anema & Klostermeyer, 1997; Crudden et al., 2005b; Holt, 1992). The storage temperature influences the dissociation of calcium ions from the casein micelles into the serum; the calcium ions were shown to decrease the catalytic efficiency of plasminogen activation (calcium acts as a competitive inhibitor of PA by increasing the Michaelis–Menten constant) (Schroeder, Nielsen, & Hayes, 2008).

## **2.7 PLASMIN ACTIVITY AND PROTEOLYSIS DETERMINATION**

Plasmin activity in milk is determined by using various methods and techniques, e.g. enzyme-linked immunosorbent assay (Collin, Compagnone, Ryba, & Baer, 1988), chromogenic/fluorogenic substrates (Hayes & Nielsen, 2000; Richardson, 1983a; Richardson & Pearce, 1981) and measurement of proteolysis using gel electrophoresis or high performance liquid chromatography (HPLC). All methods

have advantages and disadvantages and differ in specificity, sensitivity and sample preparation.

Chromogenic and fluorogenic assays are widely used to measure plasmin activity. Different types of synthetic chromogenic or fluorogenic substrates have been developed by different companies, e.g. Pefabloc PL, Spectrozyme PL and Chromozyme PL; they have lysine in the position next to the fluorogenic or chromogenic compound, e.g. coumarin or nitroaniline. Plasmin cleaves the lysine–nitroaniline bond, releasing 4-nitroaniline, which absorbs light at 405 nm and is converted into plasmin activity. The fluorogenic and chromogenic methods are sensitive and require little sample preparation to measure plasmin activity (Bastian & Brown, 1996). Less specificity and interference from other bacterial proteases, casein and whey proteins are the limitations of this method (Bastian & Brown, 1996).

In milk and milk products, hydrolysis by plasmin is measured using different techniques, e.g. PAGE, trinitrobenzenesulphonic acid (TNBS), reverse-phase (RP)-HPLC, fluorescamine etc. (Chove, Grandison, & Lewis, 2011). In the gel electrophoresis method and the RP-HPLC method, the decrease in the protein bands or the appearance of proteolysis product bands is measured. The TNBS and fluorescamine methods measure the generation of free amino groups from the hydrolysis of the protein by plasmin. Among the above methods, the fluorescamine method has a lower detection limit, HPLC is most sensitive, PAGE is the best qualitative method for monitoring the  $\beta$ -casein hydrolysis product and TNBS is simple and reliable and can be used in routine laboratory analysis (Chove et al., 2011).

## **2.8 PLASMIN IN MILK PRODUCTS**

### **2.8.1 Cheese**

Plasmin in cheese plays an important role during cheese making, especially during ripening, contributing to the quality of the cheese, e.g. ripening, flavour and texture, and reduce ripening costs (Nielsen, 2003). The effect of plasmin on curd-

forming properties remains inconclusive; however, a late lactation milk and a high SCC milk gave poor curd-forming properties in cheese making (Kelly & McSweeney, 2003). The property of the association of plasmin with the casein micelles is exploited in accelerating cheese ripening. Unlike other enzymes, the association of plasmin with casein facilitates its incorporation into curd without losing it into the whey and thus accelerates the cheese ripening process (Farkye & Fox, 1992; Kelly & McSweeney, 2003). The peptides generated by plasmin-induced hydrolysis are large and play an insignificant role in flavour development; however, other enzymes can degrade such large peptides into smaller peptides and amino acids and can generate flavouring compounds (Kelly & McSweeney, 2003). The addition of uPA was also found to increase the plasmin activity in cheeses and during ripening (Bastian et al., 1991b). Plasmin activity is lowest in Cheddar and Cheshire cheeses and higher in Swiss cheeses, e.g. Emmental, Gouda etc. (Nielsen, 2003). The higher plasmin content in Swiss cheese compared with Cheddar cheese could be due to the dissociation of some plasmin by NaCl in Cheddar curd and loss in the whey and to a higher pH range of plasmin activity in Swiss cheeses (Grufferty & Fox, 1988a; Richardson & Pearce, 1981). In addition, the heat resistance of plasmin plays a critical role; it has a high content in Swiss cheese and during ripening, whereas high cooking temperatures inactivate chymosin, other rennets, inhibitors of plasmin and PA (Bastian & Brown, 1996).

Ultrafiltration (UF) cheeses are made from ultrafiltered milk. UF influences the plasmin activity in such cheeses, because of the removal of plasmin system inhibitors in the whey and the incorporation of whey proteins. Whey proteins, especially  $\beta$ -lactoglobulin, are known to inhibit plasmin activity (Bastian, Hansen, & Brown, 1993; Chen & Ledford, 1971; Kaminogawa et al., 1972; Politis, Zavizion, Barbano, & Gorewit, 1993), which could affect plasmin activity in UF cheeses. The slow flavour development in UF cheeses is attributed to the inhibition of proteolytic enzymes (plasmin, chymosin and microbial rennet) and the binding of flavour compounds by whey proteins (Bastian et al., 1991b; Harper, Iyer, Knighton, & Lelievre, 1989; Lelievre, Creamer, & Tate, 1990; Lelievre, Iyer, Bennett, & Lawrence, 1986; Lelievre & Lawrence, 1988).

### 2.8.2 UHT milk

The plasmin-induced proteolysis in UHT milk products depends on many factors, such as processing conditions, storage conditions, level of plasmin(ogen) and its activators, and the concentration of milk and other ingredients used in the product (Nielsen, 2003). Plasminogen and its activators are heat stable, are little affected by the temperature of pasteurisation and partially survive the UHT process (140°C/4 s) (Lu & Nielsen, 1993b; Richardson, 1983a; Rollema & Poll, 1986), whereas inhibitors of the plasmin system are inactivated (Prado et al., 2006); this survival of plasminogen and its activators leads to enhanced proteolysis in heat-treated milk (Deharveng & Nielsen, 1991). UHT-treated milk systems with a pH range of 6.5–8 are a good environment for plasmin activity and the storage of such systems without refrigeration accelerates proteolysis, bitterness and age gelation because of residual plasmin activity (Chavan, Chavan, Khedkar, & Jana, 2011; Enright & Kelly, 1999; Kohlmann, Nielsen, & Ladisch, 1988, 1991).

Milk is commonly preheat treated for commercial UHT processing to minimise the bacterial load and the heat load and to stabilise the  $\beta$ -lactoglobulin (denaturation at a lower temperature will minimise deposition in the UHT plant during UHT processing) (Datta & Deeth, 2001; Newstead, Paterson, Anema, Coker, & Wewala, 2006). Newstead et al. (2006) suggested a preheat treatment of 90°C for 30 or 60 s for effective inhibition of plasmin-type proteolysis in direct UHT-processed milk. Payens (1978) suggested that the preheating of milk results in the precipitation of whey proteins on micellar surfaces, severely reduces the number of sites available for clotting and increases the gelation time (Datta & Deeth, 2001; Payens, 1978). Similar results of an increase in the gelation time were observed upon increasing the sterilisation temperature and holding time (Datta & Deeth, 2001). According to McMahon (1996), more severe heat treatment enhances chemical cross-linking within the micelles, which causes slow dissociation of the  $\beta$ -lactoglobulin- $\kappa$ -casein complex from the micelles and ultimately results in delayed gelation.

According to enzymatic mechanisms for age gelation in UHT milks, proteases (especially plasmin) are responsible for the release of the  $\beta$ -lactoglobulin- $\kappa$ -casein

complex, which eventually forms a protein network and constitutes the gel (Datta & Deeth, 2001). In the first stage, proteases cleave the peptide bonds that anchor  $\kappa$ -casein to the casein micelle and facilitate release of the  $\beta$ -lactoglobulin- $\kappa$ -casein complex. The second stage involves aggregation of the  $\beta$ -lactoglobulin- $\kappa$ -casein complex and the formation of a three-dimensional network of cross-linked proteins (Datta & Deeth, 2001; Manji & Kakuda, 1988).

Gelation of UHT milk also depends on the UHT process, i.e. direct or indirect. Milk sterilised by a direct heating method has a tendency to gel sooner during storage than milk sterilised by indirect heating (Datta & Deeth, 2001). Indirect UHT treatment has more heat intensity and proteases are inactivated to a greater extent than in direct UHT treatment (Datta & Deeth, 2001). Manji, Kakuda, and Arnott (1986) showed that directly processed UHT milk had 19% and 37% of the original plasmin and plasminogen activities, whereas indirectly processed UHT milk had no residual plasmin activity but 19% of the original plasminogen activity. As PAs are more heat stable than plasmin and plasminogen, and can activate plasminogen to increase plasmin activity, it is important to inactivate plasminogen to minimise plasmin-induced proteolysis in heat-treated products (Lu & Nielsen, 1993b).

### **2.8.3 Milk protein products**

Caseins, caseinates and whey protein concentrates and isolates are widely produced commercially and are important as functional ingredients in formulated foods, i.e. dairy, beverage, bakery dessert, pasta and confectionary products. The use of caseinates and whey proteins as functional ingredients in different products is to improve the functional properties, e.g. coagulation, gelation, hydration, foaming and emulsifying properties. Such properties can be greatly affected by the presence of plasmin and its activity (Kelly & McSweeney, 2003; Nielsen, 2003).

Acid casein has low plasmin activity, which is attributed to the low pH during manufacture, resulting in loss of plasmin into the whey; therefore, acid whey contains a large amount of plasmin. In contrast, the higher pH during the generation of sweet whey from rennet casein production results in a low plasmin level in the whey and rennet casein has high plasmin activity (Hayes & Nielsen, 2000). The slow

pH shift during lactic casein manufacture leads to a lower plasmin level, and the production of sodium caseinate under harsh processing conditions leads to a lower plasmin level (Richardson & Elston, 1984).

## **2.9 CONCLUSIONS**

This literature review consolidates published information on the plasmin system present in milk and milk products, with greater emphasis on the mechanism of plasmin's action on milk proteins. The review provides substantial information on the structural aspects of plasmin. This gives a good background for the development of a plasmin-resistant protein and for the generation of a mechanism for blocking plasmin's action on milk proteins. The review of milk proteins and their hydrolysis helps in the choice of a simple model system, method development and peptide identification, and helps in understanding the dissociation of the casein micelle upon plasmin-induced hydrolysis. The information related to the effect of different factors on plasmin activity helps in developing new hypotheses in more complex milk systems.

# Chapter 3: The mechanism of resistance to plasmin activity through protein succinylation: a model study using $\beta$ -casein

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## 3.1 INTRODUCTION

Plasmin (EC3.4.21.7) is one of the main indigenous milk enzymes that is responsible for proteolysis in milk systems (Bastian & Brown, 1996). It is part of a complex protease–protease inhibitor system. Plasminogen and its activators are heat stable, are unaffected by pasteurisation and partially survive ultra-high-temperature (UHT) treatment (Nielsen, 2002). The high heat stability allows activation of plasminogen to plasmin during product storage and causes breakdown of the proteins in various milk products, e.g. UHT milks and beverages, and affects the flavour and texture of these products (Nielsen, 2002). It is thus critical to control plasmin's activity in these milk products.

Plasmin is a serine protease that specifically hydrolyses Lys–X and Arg–X bonds, with a preference for Lys–X bonds. Plasmin is similar to trypsin in activity but with greater specificity (Bastian & Brown, 1996). Therefore, it could be hypothesised that plasmin-induced hydrolysis can be minimised by decreasing the availability of lysine residues through chemical modification. In previous studies, the formation of lactulosyl-lysine through lactosylation was found to affect plasmin-induced hydrolysis negatively (Dalsgaard, Holm Nielsen, & Bach Larsen, 2007). However, because of the complexity of the reaction and the involvement of different stages of the Maillard reaction, along with the cross-linking, it was difficult to understand the exact mechanism. Therefore, a study using a simpler method of substrate modification with minimum side reactions was needed.

Modification of the lysine residues of milk proteins is used extensively as a means to alter and to study their physicochemical and functional properties, namely increased aqueous solubility, altered viscosity and modified surfactant properties

such as emulsification and foaming (Ahn, 1988; Kim, Choi, & Hong, 1997; Nakai & Li-Chan, 1989; Schwenke, Rauschal, Linow, & Pähz, 1981; Strange, Holsinger, & Kleyn, 1993).

Succinylation modifies the lysine residue on a protein through covalent attachment of an anionic succinate residue to its  $\epsilon$ -amino group (Strange et al., 1993). The succinylation of protein converts a cationic amino group (positively charged) to an anionic residue (negatively charged) (Franzen & Kinsella, 1976) and increases the negative charge, leading to important changes in electrostatic interactions (Alford et al., 1984). The increased electrostatic repulsive forces lead to extensive unfolding and expansion of the polypeptide chain and/or dissociation of aggregated or polymeric proteins (Klotz & Keresztes-Nagy, 1962; Kumagai, 2012; Strange et al., 1993). These alterations in the physicochemical properties of proteins and the increase in solubility of succinylated proteins have important functional effects (Beuchat, 1977; Chen, Richardson, & Amundson, 1975; Franzen & Kinsella, 1976; Habeeb, Cassidy, & Singer, 1958; Strange et al., 1993). Succinylation of  $\beta$ -casein increases the net negative charge of the modified casein and prevents its aggregation and precipitation in the presence of high levels of  $\text{Ca}^{2+}$  ions (Hoagland, 1966; Strange et al., 1993).

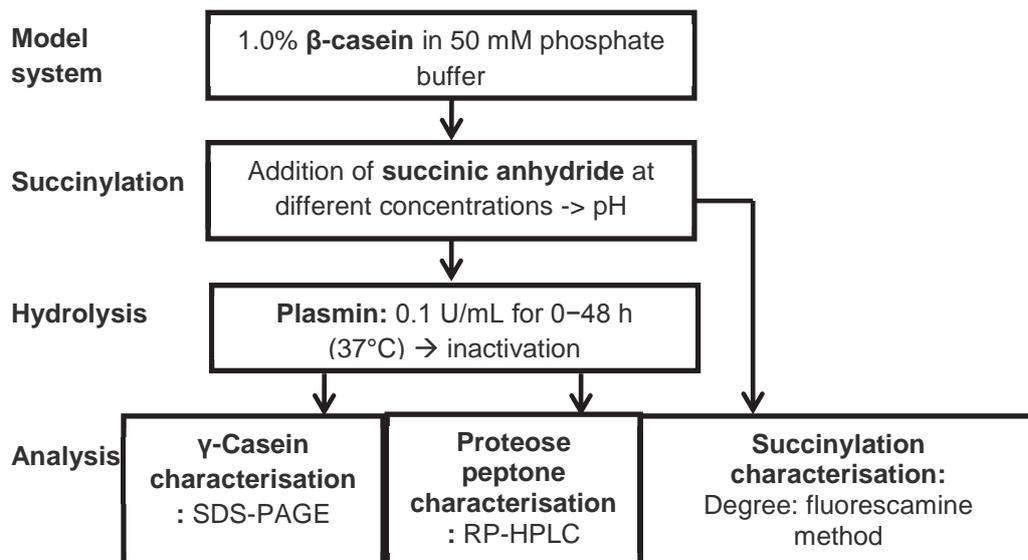
The effect of the succinylation of protein on its hydrolysis by different enzymes was found to be affected by the type of protein, the extent of protein modification and the type of protease (Groninger & Miller, 1979). Succinylation of protein negatively affected its hydrolysis by trypsin (Groninger & Miller, 1979; Li & Bertsch, 1960; Woods & Kinsella, 1980), whereas it had no effect on or increased its hydrolysis by pepsin. The rates of hydrolysis of succinylated proteins by  $\alpha$ -chymotrypsin and pepsin were found to increase, which was attributed to the effect of the alteration in the protein conformation by succinylation on the reaction kinetics (Alford et al., 1984). Groninger and Miller (1979) observed that in vitro trypsin hydrolysis was less for succinylated fish protein. Li and Bertsch (1960) found that the only peptide bonds that were cleaved by trypsin in tryptic digests of succinyl-adrenocorticotropins were those containing arginine, in accordance with the enzymatic specificity of trypsin. Matoba and Doi (1979) observed that succinylated

casein was considerably more resistant than untreated casein to hydrolysis by trypsin.

The present work was undertaken to study the effect of different degrees of succinylation on the plasmin-induced hydrolysis of milk proteins, along with the site-wise specificity for succinylation and plasmin-induced hydrolysis, to understand the mechanism of the decrease in the hydrolysis.

## 3.2 MATERIALS

Pure bovine  $\beta$ -casein [ $> 95\%$  purity, as measured by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)] was made using in-house technology at the Fonterra Research and Development Centre and was used as a substrate. Plasmin from bovine plasma (5 U/mL) was purchased from Roche/Boehringer Mannheim GmbH (Mannheim, Germany). The chemicals for the SDS-PAGE and reverse-phase high performance liquid chromatography (RP-HPLC) buffers were purchased from Merck (Darmstadt, Germany). An acrylamide/bisacrylamide mixture 37.5:1 (2.6% C) from Bio-Rad (Hercules, CA, USA) was used. Water-free acetone was from Thermo Fischer Scientific (Auckland, New Zealand). Succinic anhydride and fluorescamine (4-phenylspiro[furan-2(3H),19-phthalan]-3,39-dione) were purchased from Sigma–Aldrich (Auckland, New Zealand).



Scheme 1: Experimental plan.

## 3.3 METHODS

### 3.3.1 Succinylation of $\beta$ -casein

The  $\beta$ -casein model system was prepared by dissolving 1% (w/w)  $\beta$ -casein in 50 mM sodium phosphate buffer at pH 6.7 to mimic the concentration of  $\beta$ -casein in the milk system. Succinic anhydride was added to the  $\beta$ -casein model system at room

temperature (20°C) such that different degrees of succinylation, i.e. 0, 20, 30, 40 and 50%, were achieved. The pH was kept constant at 6.7 by the addition of 1% NaOH.

### **3.3.2 Measurement of the degree of succinylation**

The decrease in the level of reactive amino groups on the protein due to succinylation was used as a measure of the degree of succinylation and was determined using the fluorescamine method as described by Bhatt et al. (2014). The fluorescence intensities were then measured using a FluoroMax-4 compact spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ, USA) at excitation and emission wavelengths of 390 and 475 nm respectively and at a bandwidth of 5 nm. All measurements were made in triplicate.

### **3.3.3 RP-LC–MS/MS analysis of the succinylated $\beta$ -casein**

The sites for the succinylation of  $\beta$ -casein were identified using a reverse-phase liquid chromatography–tandem mass spectrometry (RP-LC–MS/MS) technique, in which samples were first hydrolysed by trypsin and then analysed using a mass spectrometer.

#### ***Tryptic digestion of the proteins***

$\beta$ -Casein samples with different degrees of succinylation were digested with trypsin in 0.2 M ammonium bicarbonate buffer (pH 8.1) at 37°C for 4 h, using an enzyme:substrate ratio of 1:25 (w/w), followed by inactivation of the trypsin with the addition of 10  $\mu$ L of 50% formic acid.

#### ***RP-LC–MS/MS analysis***

The hydrolysed proteins were analysed by RP-LC–MS/MS using an LTQ Orbitrap mass spectrometer (Thermo Finnigan, West Palm Beach, FL, USA) equipped with an electrospray ionisation (ESI) source and a U3000 LC system (Dionex/LC Packings, Hercules, CA, USA) in capillary flow mode. A sample (5  $\mu$ L) was loaded on to a capillary C18 PepMAP column (150 mm x 300  $\mu$ m internal diameter, 300 Å, 3  $\mu$ m particle size, Dionex/LC Packings), using a pre-concentration/desalting step on a capillary PepMAP trap (300  $\mu$ m internal diameter x 1 mm). The sample was loaded

on to the trap at 30  $\mu\text{L}/\text{min}$  using 0.1% formic acid/0.1% trifluoroacetic acid (TFA) in water (v/v). After 5 min, the trap was switched in-line with the capillary column and the peptides were separated at 3  $\mu\text{L}/\text{min}$  using a binary buffer system (0.2% formic acid in acetonitrile and 0.2% formic acid in water) and a 45 min gradient (4.5% acetonitrile for 7 min, 4.5–36% over 33 min and 36–81% over 5 min). Information-dependent acquisition experiments were performed on the mass spectrometer according to the following parameters: full scan ( $m/z$  400 to 2000) in the Orbitrap using 30,000 resolutions, followed by MS/MS in the LTQ of the top six most intense peaks in the full scan. Former target ions were excluded for 20 s.

### ***Protein identification***

Protein identification for succinylation was done using MS/MS spectral data matching (Mascot search engine version 2.2.04, Matrix Science, London, UK) against an updated National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) non-redundant sequence database (NCBI nr) by ESI-MS/MS peptide mass fingerprinting experiments. A mass tolerance value of 10 ppm, trypsin as the proteolytic enzyme, serine phosphorylation and methionine oxidation were used as a variable modification. An error tolerance search was performed to determine lysine-succinylation (100.02 Da) modification.

### **3.3.4 Plasmin-induced hydrolysis**

The succinylated  $\beta$ -casein model system was hydrolysed by adding plasmin at 15  $\mu\text{L}/\text{mL}$  (0.1 U/mL) of the 1%  $\beta$ -casein system incubated in a water bath at 37°C. The hydrolysis pattern was observed at incubation times of 0, 0.25, 0.5, 1, 2, 4, 8, 24 and 48 h. Samples were removed at each incubation time and the reaction was stopped immediately by adding 50  $\mu\text{L}$  of SDS (2% w/w). The inhibitory effect of SDS (2%) on plasmin activity has been described previously (Bhatt et al., 2014).

### **3.3.5 SDS-PAGE**

To study the effect of succinylation on proteins and the hydrolysis pattern of proteins, SDS-PAGE was performed as described by Anema and Klostermeyer (1997). For thorough analysis, the extent and the rate of hydrolysis were measured

by indirect measurement of the hydrolysis product  $\gamma$ -casein ( $\gamma_2 + \gamma_3$ ) and its further hydrolysis. Identification of  $\gamma$ -casein elution pattern on SDS-PAGE is shown in Appendix 3.

Hydrolysed samples were dispersed in SDS sample buffer [0.5 M Tris–HCl buffer, pH 6.6, containing 2% (w/v) SDS, 0.2% lactoferrin] in a 1:10 ratio (v/v). Prepared sample (10  $\mu$ L) was loaded per well of the SDS-PAGE system. The gels were run at 210 V and 70 mA for approximately 1 h. After electrophoresis, the gels were stained using a staining solution [0.1% (w/v) Amido Black 10B in 10% (v/v) acetic acid and 25% (v/v) isopropanol] for 1 h and then destained using 10% (v/v) acetic acid solution until a clear background was achieved. The gels were scanned using a scanner (GE-Image Scanner III, GE Healthcare Ltd, New Zealand) and the protein bands on the gel were quantified using ImageQuant TL (v 2005) software to give a numerical protein–dye absorbance value. Lactoferrin was used as a standard to compensate for the gel-to-gel differences in the absorbance values.

### **3.3.6 RP-HPLC**

#### ***Preparation of pH 4.6 extracts***

pH-4.6-soluble fractions were prepared by lowering the pH of hydrolysed samples to pH 4.6 with 0.5 M sodium acetate buffer, and allowing the mixtures to stand for 1 h at room temperature. The mixtures were centrifuged at 14,000 rev/min for 15 min and the supernatants were collected to yield the pH 4.6 filtrates.

#### ***Analysis of peptides by RP-HPLC***

HPLC analysis was performed on Agilent 1100 series equipment with a UV/VIS photodiode array multi-wavelength detector using a 250 mm x 4.6 mm reversed-phase column Phenomenex-Jupiter 300 C18, 5  $\mu$ m (Phenomenex, Torrance, CA, USA) at 40°C and a binary solvent gradient system at a flow rate of 1 mL/min and detection at 210 nm. Solvent A: 0.1% (v/v) TFA in Milli-Q water and solvent B: 0.1% (v/v) TFA in HPLC-grade acetonitrile were used. The proportion of solvent B was increased from 15 to 35% during the first 20 min and, after 5 min, increased to 65% in 35 min and, after 5 min, finally decreased back to 15% solvent B in 5 min in readiness for the next sample injection. Injections of 50  $\mu$ L of filtrates were made by

auto-injector. All samples were analysed in duplicate and data analysis was computed by Agilent (Hewlett Packard) Chemstation software (Hewlett Packard, Palo Alto, CA, USA). The peptides were identified as proteose peptones (PP5, PP8slow and PP8fast) and were quantified by integration of peak areas. Identification and elution pattern of proteose peptone fractions and hydrolysis analysis using RP-HPLC is explained in Appendix 4.

### 3.4 RESULTS AND DISCUSSION

#### 3.4.1 Characterisation of succinylated $\beta$ -casein samples

Succinylation involves the attachment of a succinate (carboxylate) group at the  $\epsilon$ -amino group of lysine on a protein. The  $\beta$ -casein system was modified by adding different concentrations of succinic anhydride to obtain five different degrees of succinylation. It was observed that, with an increase in the amount of succinic anhydride, the percentage of reactive amino groups decreased and this was used as a measure of the degree of succinylation (%) (Table 3).

Table 3. Degree of succinylation of  $\beta$ -casein

Sample	Fluorescamine absorbance (average)	Free amino groups (%)	Succinylation (%)
Control	638.3	100.0	0.0
S1	499.7	78.3	21.7
S2	441.0	69.1	30.9
S3	393.3	61.6	38.4
S4	321.7	50.4	49.6

Succinylation sites on the  $\beta$ -casein backbone were identified using LC-MS/MS analysis after hydrolysis by trypsin. Trypsin was used to generate smaller peptides containing one or two lysine residues; modified lysine residues are not recognisable by trypsin, which was the basis for the analysis. In the MS/MS analysis, succinylated peptides containing Lys28, Lys29, Lys32, Lys99, Lys105 and Lys107 as the C-terminal residues were absent.  $\beta$ -Casein is unstructured (mostly random coil) in solution, which facilitates steric exposure of the  $\epsilon$ -amino group of lysine; in addition, the  $\epsilon$ -amino group has a relatively low  $pK_a$  value (Franzen & Kinsella, 1976), allowing it to undergo succinylation readily. Only six of the 11 lysine residues were succinylated, i.e. Lys28, Lys29, Lys32, Lys99, Lys105 and Lys107 (Table 4). All were present in the PP5 region (N-terminal side) of  $\beta$ -casein, which could have been due to the accessibility of the residues in the hydrophilic region.

Sites for succinylation have not been determined previously; however, lactosylation is a reaction that involves the attachment of lactose on the same  $\epsilon$ -amino group of

lysine on proteins in the milk system. Scaloni et al. (2002) identified Lys32, Lys48, Lys107, Lys113 and Lys176 as being the lactosylation sites, which are different from the succinylation sites identified in the present study. This means that the specificity differs from reaction to reaction, which could be due to the different system (casein micelle in milk versus  $\beta$ -casein in the model system) or to the changes in the structure and reactivity of different residues with heat treatment, in the case of lactosylation. Scaloni et al. (2002) used milk for site identification. In the present study, pure  $\beta$ -casein was used, which could have facilitated easier access to the lysine residues.

Table 4. RP-LC-MS/MS analysis of  $\beta$ -casein for site identification of succinylation following digestion with trypsin. As succinylated residues are not hydrolysed by trypsin, the absence of peptides containing these residues at the C terminus was the basis of the analysis

$\beta$ -Casein succinylation site	Sequence*	Additional modification site	Charge	Mass	
				Expected	Calculated
K28, K29	$\beta$ -CN(26–32): R.INK*K*IEK.F		2	1071.59	1071.58
K32	$\beta$ -CN(29–48): K.KIEK*FQSEEQQTDELQDK.I	SER P 35	3	2579.21	2579.19
K29, K32	$\beta$ -CN(29–48): K.K*IEK*FQSEEQQTDELQDK.I	SER P 35	3	2759.20	2759.17
K32	$\beta$ -CN(30–48): K.IEK*FQSEEQQTDELQDK.I	SER P 35	2	2531.07	2531.06
K99	$\beta$ -CN(98–105): K.VK*EAMAPK.H		2	972.50	972.49
K99, K105	$\beta$ -CN(98–107): K.VK*EAMAPK*HK.E		3	1337.67	1337.66
K105	$\beta$ -CN(100–107): K.EAMAPK*HK.E		3	1010.49	1010.49
K105, K107	$\beta$ -CN(100–113): K.EAMAPK*HK*EMPFK.Y		3	1839.87	1839.85
K107	$\beta$ -CN(106–113): K.HK*EMPFK.Y		4	1112.54	1112.53

\* Peptide sequences are from the B variant of  $\beta$ -casein. In this table, only the peptides giving fruitful information regarding sequence coverage and/or modified site localisation are included.

**K\*** Succinyl-lysine.

### 3.4.2 Effect of succinylation of $\beta$ -casein on its plasmin-induced hydrolysis

$\beta$ -Casein samples with different degrees of succinylation were hydrolysed by plasmin, as described in Section 3.3.4. Plasmin-induced hydrolysis of  $\beta$ -casein leads to the generation of  $\gamma$ -casein, proteose peptones and smaller peptides.

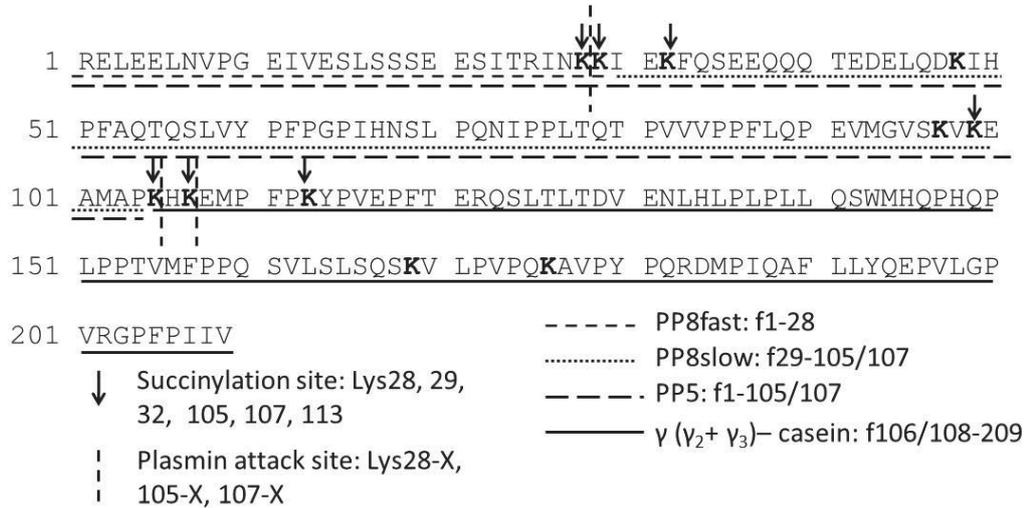


Figure 13.  $\beta$ -Casein succinylation sites as identified by LC-MS/MS analysis. Succinylation sites and plasmin cleavage sites are represented in the schematic, along with the peptides generated after hydrolysis by plasmin.

All the identified succinylation sites except Lys113 were found to be present in the hydrophilic block (N1-105/107) of  $\beta$ -casein (Figure 13). This hydrophilic block forms proteose peptone fractions after plasmin-induced hydrolysis. Therefore, it was hypothesised that succinylation has an effect on the further hydrolysis of proteose peptones but not on  $\gamma$ -casein. Thus, for thorough analysis and better understanding of the formation and degradation of  $\gamma$ -casein and proteose peptones, two complementary methods, RP-HPLC for proteose peptones and SDS-PAGE for  $\gamma$ -casein, were used and the entire hydrolysis pattern was divided into these two stages.

1. Primary stage: Hydrolysis of substrate  $\beta$ -casein and generation of  $\gamma$ -casein and proteose peptones, measured by SDS-PAGE and RP-HPLC respectively.
2. Secondary stage: Further hydrolysis of the  $\gamma$ -casein and proteose peptones generated into smaller peptides, measured as a decrease in concentration by SDS-PAGE and RP-LC respectively.

### ***γ-Casein characterisation***

With an increase in succinylation, there was a decrease in the generation of  $\gamma$ -casein, as observed using SDS-PAGE (Figure 14), which indirectly represented the decrease in  $\beta$ -casein hydrolysis. A decrease in the generation of  $\gamma$ -casein, i.e. the primary stage, and a decrease in the peak (maximum) concentration of  $\gamma$ -casein were observed with an increase in the degree of succinylation (Figure 15).

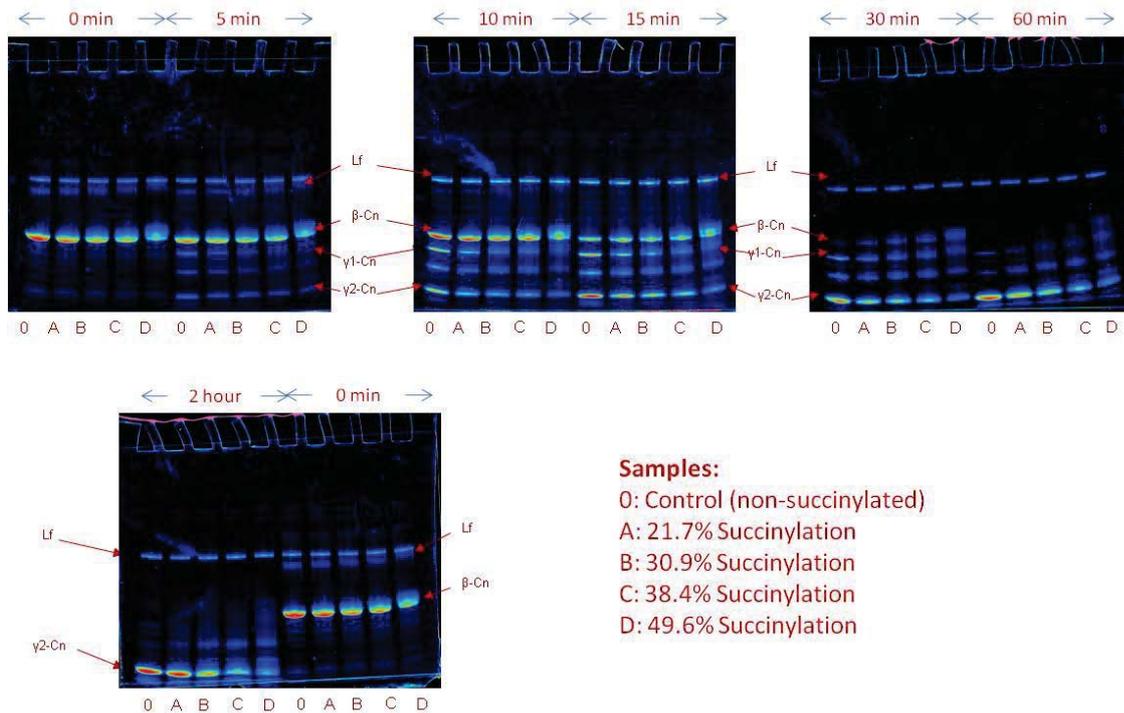


Figure 14. Typical SDS-PAGE gel for the plasmin-induced hydrolysis.  $\beta$ -Casein samples with different degrees of succinylation were hydrolysed by plasmin and analysed by quantification of  $\gamma$ -casein using gradient (4–15%) SDS-PAGE. Amido Black was used for staining and quantification was performed using ImageQuant software. The succinylated samples were hydrolysed for 0 (no hydrolysis), 5, 10, 15, 30, 60 and 120 min. Lf: lactoferrin,  $\beta$ -Cn:  $\beta$ -casein,  $\gamma$ 1-Cn:  $\gamma$ 1-casein,  $\gamma$ 2-Cn:  $\gamma$ 2-casein.

The decrease in the rate of hydrolysis with an increase in succinylation followed a linear trend ( $R^2 = 0.9467$ ) and the rate of decrease was  $-1.184$ , indicating the negative effect of succinylation on plasmin-induced hydrolysis (Figure 20), based on the generation of  $\gamma$ -casein.  $\gamma$ -Casein and proteose peptones were generated by the hydrolysis of  $\beta$ -casein at Lys105 and Lys107; upon succinylation, these residues become succinylated, as observed in the RP-LC-MS/MS analysis (Table 4), making them resistant to plasmin-induced hydrolysis.

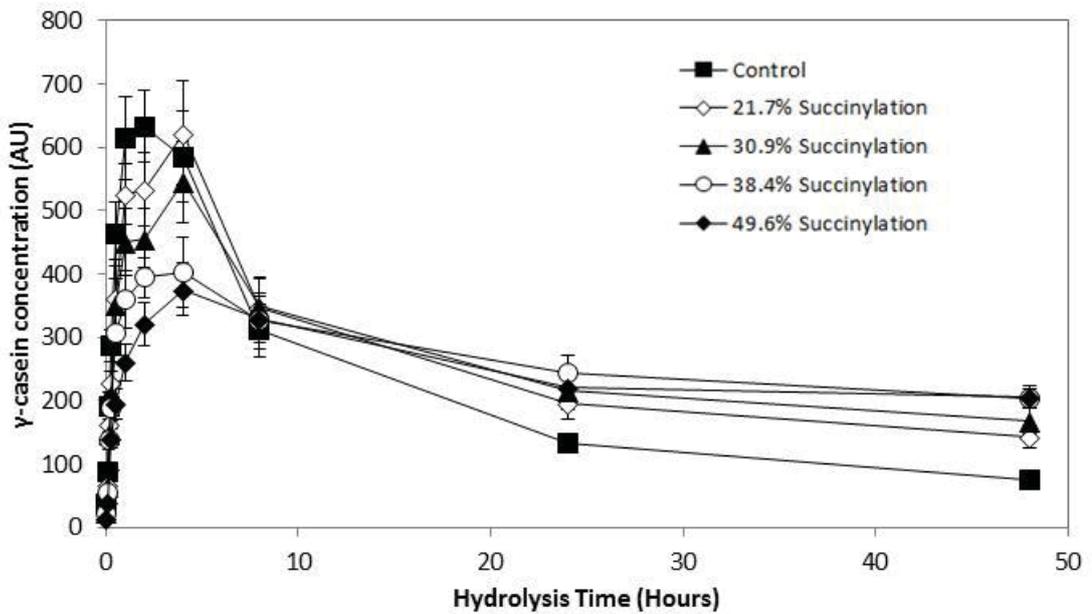
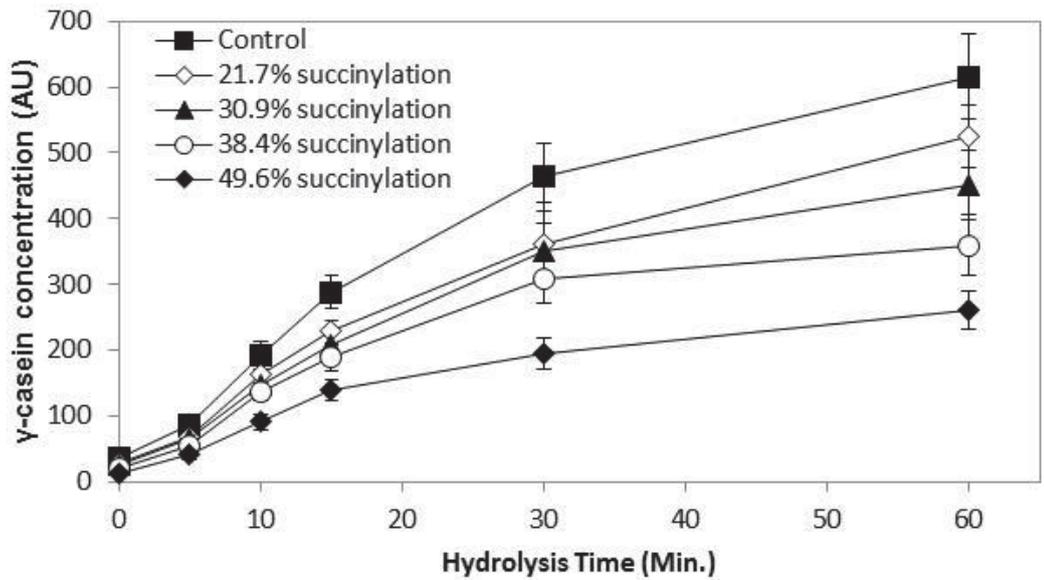


Figure 15. Effect of the succinylation of  $\beta$ -casein on its hydrolysis by plasmin, as measured by quantification of the hydrolysis product, i.e.  $\gamma$  ( $\gamma_2 + \gamma_3$ )-casein, A. 60 min hydrolysis pattern B. 48 h hydrolysis pattern, using SDS-PAGE. Error bars refer to standard deviations.

The peak  $\gamma$ -casein concentration was related to the maximum amount of substrate  $\beta$ -casein that was hydrolysed by plasmin. The greatest peak concentrations occurred for the control samples and the peak concentration decreased with an

increase in the degree of succinylation (Figure 15). There was also a shift in the peak concentration upon succinylation (Figure 15). The control sample reached the peak  $\gamma$ -casein concentration after only 2 h of hydrolysis and the concentration then decreased with further hydrolysis. In contrast, succinylated samples reached peak concentrations after 4 h of hydrolysis.

The hydrolysis reaction was monitored continuously for 48 h. The  $\gamma$ -casein concentration for the control and the less succinylated (i.e. 21.7 and 30.9%) samples decreased at a faster rate than that for the more succinylated samples. After 8 h of hydrolysis, all samples had similar  $\gamma$ -casein concentrations and followed a similar trend; this was attributed mainly to non-succinylation of the  $\gamma$ -casein residues (from the RP-LC–MS/MS analysis in Table 4), such that they did not become resistant to further hydrolysis.

### ***Proteose peptone characterisation***

Proteose peptones are the hydrophilic hydrolysis products, e.g. PP5,  $\beta$ -casein f(N–105/107) (Andrews, 1978a; Eigel, 1981), PP8fast,  $\beta$ -casein f(N–28) (Andrews, 1978b) and PP8slow,  $\beta$ -casein f(29–105/107) (Andrews & Alichanidis, 1983), of  $\beta$ -casein that are heat stable and acid soluble at pH 4.6 (Kolar & Brunner, 1969, 1970).

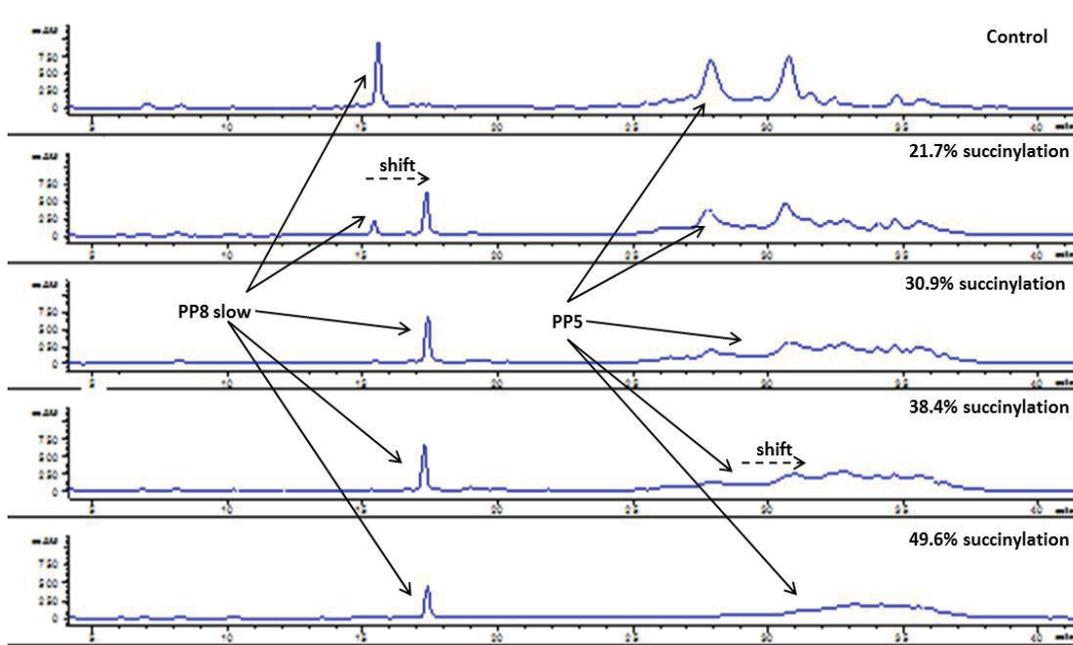


Figure 16. Hydrolysis pattern observed using RP-HPLC after 2 h of hydrolysis. With an increase in the degree of succinylation, the elution patterns for PP5 and PP8slow changed and they eluted later. The shifts in the peaks were due to succinylation of PP8slow and PP5, as observed in the RP-LC-MS/MS analysis (Table 4).

Peptide mapping and quantification of both succinylated samples and control (non-succinylated) samples enabled the effect of succinylation on plasmin-induced hydrolysis to be determined. The elution times for PP5 and PP8slow shifted in succinylated samples (Figure 16). This was attributed mainly to a change in the charge and size of the protein because of the carboxylate groups that were added by succinylation at Lys28, Lys29, Lys32, Lys99 and Lys105, as observed by RP-LC-MS/MS (Table 4), leading to a more heterogeneous population of proteins (Hoagland, 1966; Ma et al., 2009).

The hydrolysis patterns of different succinylated samples, as shown in Figure 17, clearly indicated that succinylation decreased the proteose peptone concentration, which resulted from the decrease in the hydrolysis of succinylated  $\beta$ -casein.

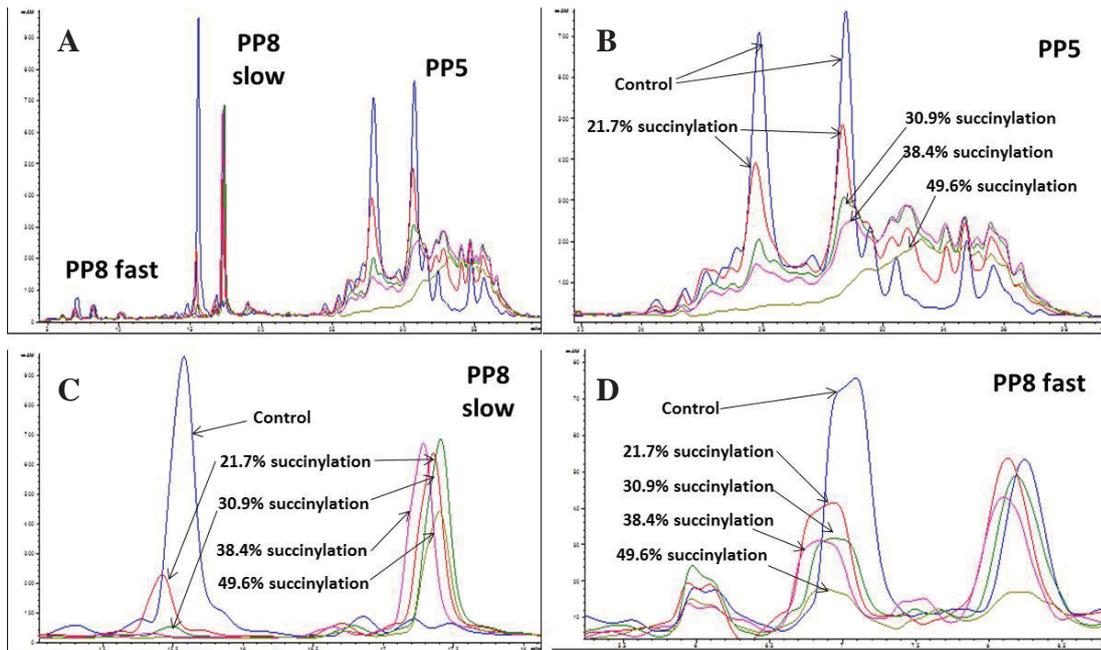


Figure 17. Hydrolysis patterns observed using RP-HPLC (overlays) after 2 h of hydrolysis: A, total proteose peptones; B, PP5; C, PP8slow; D, PP8fast. Succinylation negatively affected the plasmin-induced hydrolysis, as observed by the changes in the elution patterns of the peptides and the smaller peaks resulting from reduced peptide formation.

In the primary stage of the hydrolysis reaction, PP5 and PP8slow increased linearly but the trend for PP8fast was not very clear (Figure 18), which could have been due to its low concentration and its rapid further hydrolysis into smaller peptides. The total concentration of proteose peptones (PP5 + PP8slow + PP8fast) increased linearly because PP5 and PP8slow were the main contributors in the profile of total proteose peptones.

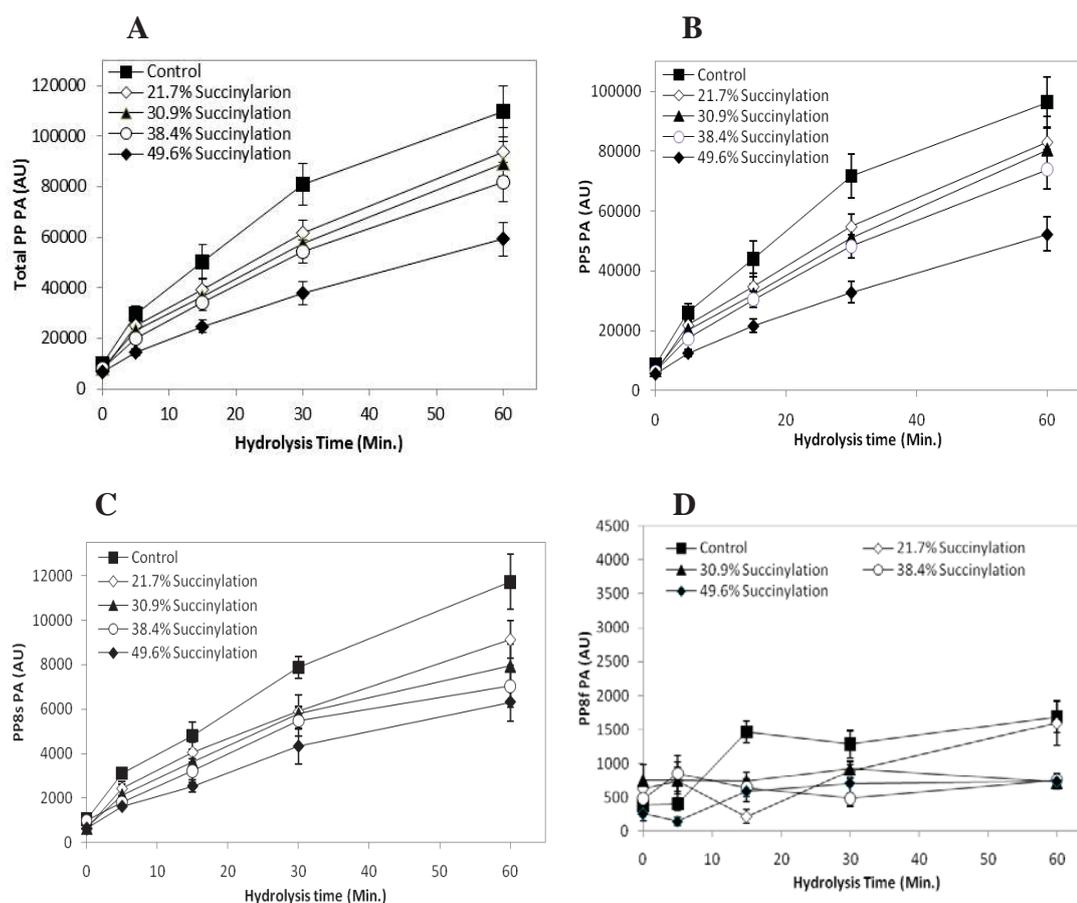


Figure 18. Comparative hydrolysis patterns, as observed from RP-HPLC quantification: A, total proteose peptones; B, PP5; C, PP8slow; D, PP8fast. The areas under the peaks for different peptides were calculated and are presented as an increase in the peptide concentration (PA – area under peak; AU – arbitrary units). Note the change in scale. Error bars refer to standard deviations.

The rate of plasmin-induced hydrolysis of  $\beta$ -casein was indirectly measured from the tangent of the generation of total proteose peptones during the first 60 min of hydrolysis and was found to decrease linearly ( $R^2 = 0.917$ ) with an increase in the degree of succinylation (Figure 20), indicating that succinylation of  $\beta$ -casein increased its resistance to plasmin-induced hydrolysis.

The effect of succinylation was quite clear in the primary stage of the hydrolysis because  $\beta$ -casein was the primary target of plasmin. However, the effect of succinylation became complicated in the later/secondary stage of the hydrolysis when no more  $\beta$ -casein was available.

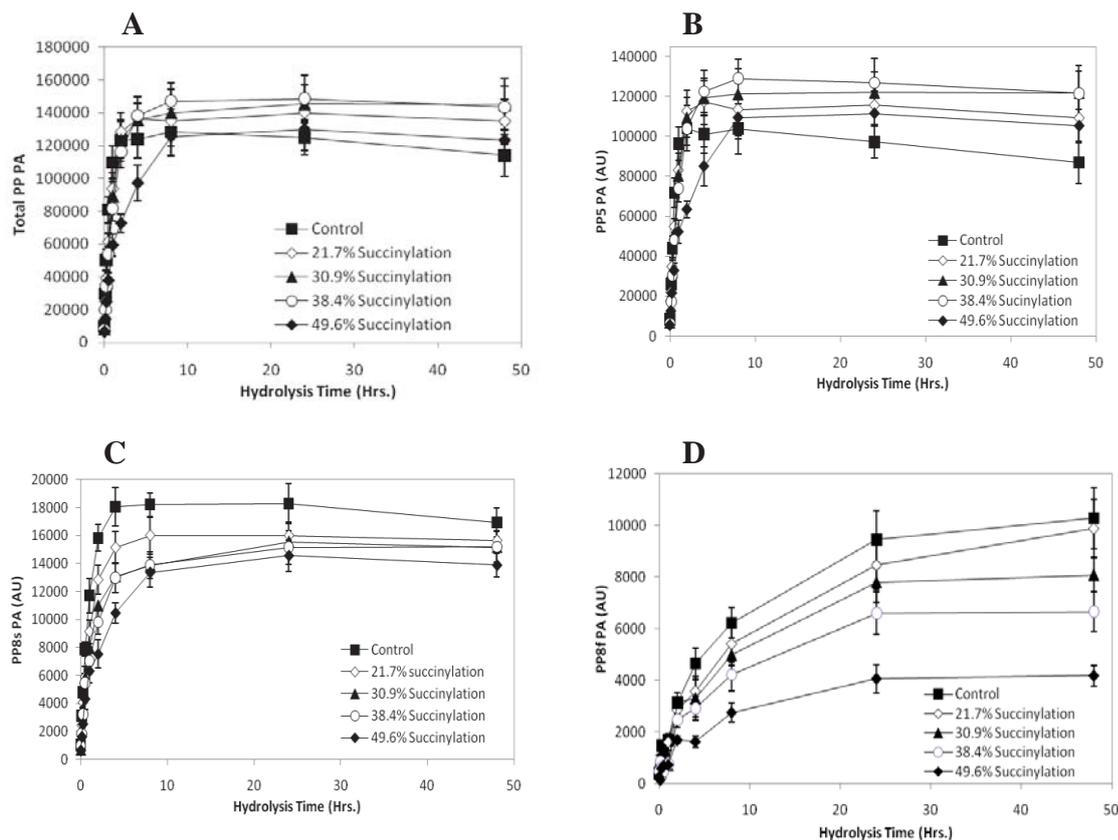


Figure 19. Complete hydrolysis patterns, as observed from RP-HPLC quantification: A, total proteose peptones; B, PP5; C, PP8slow; D, PP8fast. The areas under the peaks for different peptides were calculated and are presented as an increase in the peptide concentration (PA – area under peak; AU – arbitrary units). Note the change in scale. Error bars refer to standard deviations.

The complete hydrolysis patterns over 48 h were very different for succinylated samples and control samples. It can be seen from this analysis that succinylation affected the complete hydrolysis pattern. In the initial stage of the plasmin-induced hydrolysis, the rate of hydrolysis of the substrate  $\beta$ -casein was higher in control samples and decreased linearly with an increase in succinylation at Lys28, Lys29, Lys32, Lys99, Lys105 and Lys107 (Table 4), making it resistant to hydrolysis by plasmin. However, the pattern changed in the secondary stage, because the substrate PP5 followed a similar trend to that of the substrate  $\beta$ -casein in the primary stage. In the secondary stage, PP5 was resistant to plasmin-induced hydrolysis because of succinylation at Lys28, Lys29 and Lys32 (Table 4), and the concentration of PP5 after 2 h of hydrolysis was higher in succinylated samples than in control samples (Figure 19B). Succinylation of PP5 made its degradation reaction slower than that of the control samples. Therefore, the PP5 concentration increased

continuously in the more succinylated samples, whereas, because PP5 was hydrolysed to PP8slow, PP8fast and other peptides, the PP5 concentration decreased in the control and less succinylated samples (Figure 19). As PP5 was the main contributor to the total proteose peptones, the total proteose peptone trend more or less followed the PP5 trend. Thus, with respect to the effect of succinylation, the trend in the secondary stage of hydrolysis was very different from that in the primary stage.

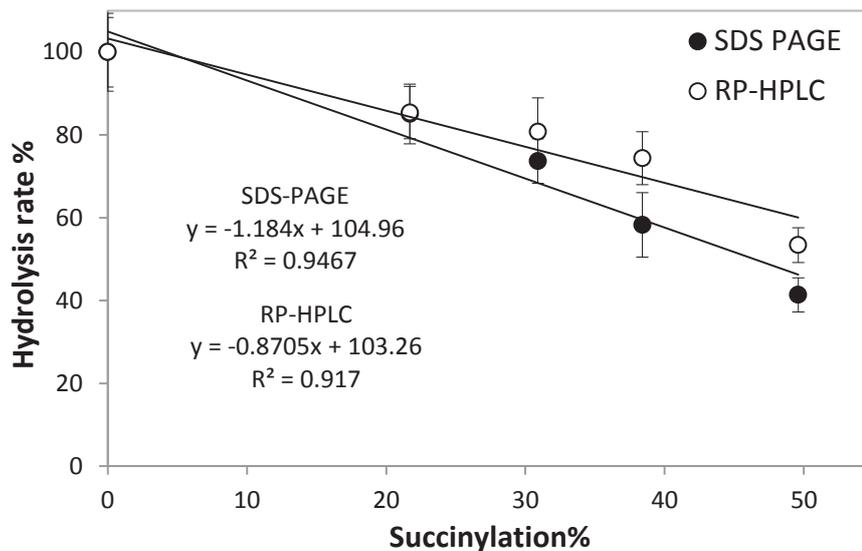


Figure 20. Effect of succinylation on the rate of  $\beta$ -casein hydrolysis by plasmin. The  $\beta$ -casein hydrolysis rate was calculated from the tangent of the generation of  $\gamma$ -casein (as measured by SDS-PAGE, Figure 15) and total proteose peptones (as measured by RP-HPLC, Figure 18). Error bars refer to standard deviations.

The decrease in the initial hydrolysis rate was  $-0.8705$  based on the generation of proteose peptones, but was  $-1.184$  based on the generation of  $\gamma$ -casein. This difference was attributed mainly to measurement of the different peptides and their different behaviours during plasmin-induced hydrolysis. As observed in Section 3.4.1, all succinylation sites occur in the proteose peptones (Table 4 and Figure 13) rather than in  $\gamma$ -casein, which could result in much slower hydrolysis of the proteose peptones. In addition,  $\gamma$ -caseins are hydrophobic peptides and tend to aggregate through hydrophobic interactions, making them less accessible and/or quite resistant to plasmin attack; in contrast, proteose peptones are soluble peptides and have multiple plasmin-accessible sites, making them a favourable

plasmin target. These properties of the peptides caused the differences in the analysis because we were measuring the products of a dynamic hydrolysis reaction.

### 3.5 PROPOSED MECHANISM

Because plasmin hydrolyses  $\beta$ -casein at Lys28, Lys105 and Lys107 and succinylation also targets these lysine residues, as observed by RP-LC–MS/MS analysis (Table 4), succinylation caused a decrease in plasmin-induced peptide formation. The substrate  $\beta$ -casein became resistant to plasmin because its availability to the enzyme for hydrolysis was decreased by the formation of succinyl-lysine through succinylation (Figure 21).

The effect of succinylation on the availability of a substrate to plasmin has not been studied previously. However, the current findings can be related to earlier studies on the hydrolysis of succinylated proteins by different enzymes. Plasmin is a trypsin-like serine protease that has identical specificity to trypsin and hydrolyses proteins at lysine and arginine residues. Groninger and Miller (1979) observed that in vitro trypsin hydrolysis was less for succinylated fish protein. Li and Bertsch (1960) found that the only peptide bonds that were cleaved by trypsin in tryptic digests of succinyl-adrenocorticotropins were those containing arginine, in accordance with the enzymatic specificity of trypsin. Matoba and Doi (1979) observed that succinylated casein was considerably more resistant to hydrolysis by trypsin than untreated casein.

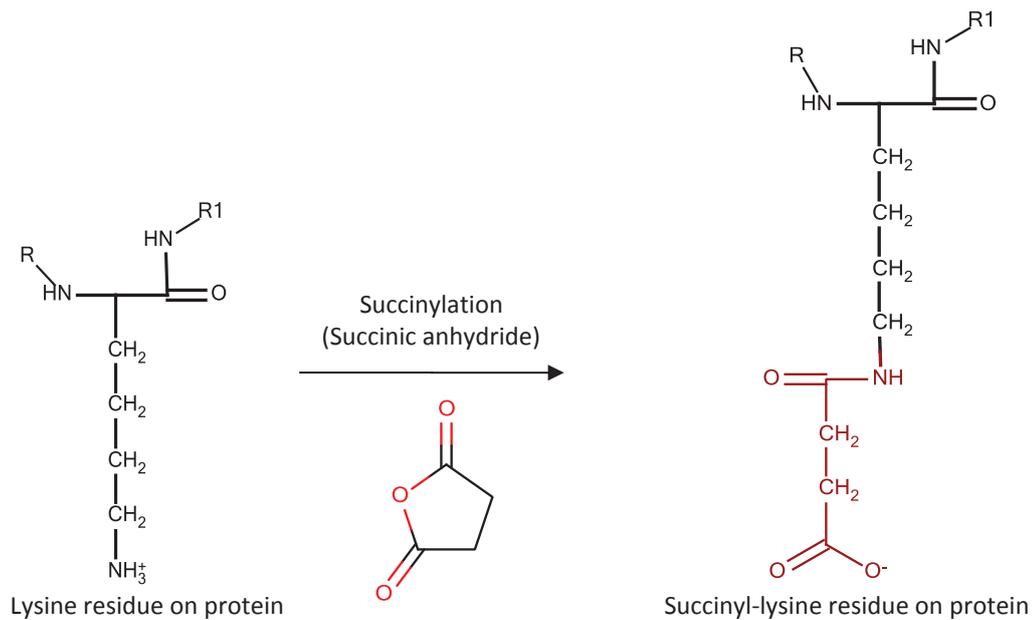


Figure 21. Succinylation modifies lysine residues.

As outlined earlier, plasmin has specificity towards Lys–X and Arg–X bonds, and has a preference for Lys–X bonds (Bastian & Brown, 1996). The positioning of the substrate and its binding in the substrate-binding pocket of the serine protease is the first and a critical step in the hydrolysis mechanism of serine proteases. The substrate specificity of serine protease is driven by a residue located at the bottom of the enzyme’s specificity pocket (Perona & Craik, 1995). In trypsin-like serine proteases, the aspartic acid residue is located at the bottom of the specificity/substrate-binding pocket (Figure 22) (Whitaker, 2002). In human plasmin, the side chain of Asp735 is located at the bottom of the S1 specificity pocket, which creates a negatively charged S1 site and accounts for its specificity towards the positively charged P1 (lysine) residues of the substrate, especially lysine or arginine (Krieger et al., 1974; Wang et al., 2000). The carbon ion of the scissile bond becomes positioned near the nucleophilic serine of the catalytic triad (Perona & Craik, 1995). The catalytic triad of the serine protease trypsin is His57, Asp102 and Ser195, and that of bovine plasmin is His598, Asp641 and Ser736 (Schaller et al., 1985).

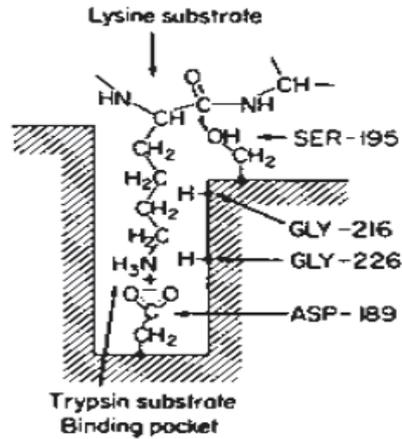


Figure 22. Substrate-binding site of trypsin (Whitaker, 2002).

Different serine protease sequences, i.e. human plasminogen, bovine plasminogen, human trypsin and bovine chymotrypsin, were matched to generate a hypothesis based on sequence homology (Figure 23). As expected, the residues of the catalytic triad were the same, i.e. aspartic acid, serine and histidine, in all serine proteases, and the residue located at the bottom of substrate-binding pocket, which determines specificity, was the same, i.e. aspartic acid, in all serine proteases except for chymotrypsin, explaining the specificity for positively charged lysine and arginine residues.

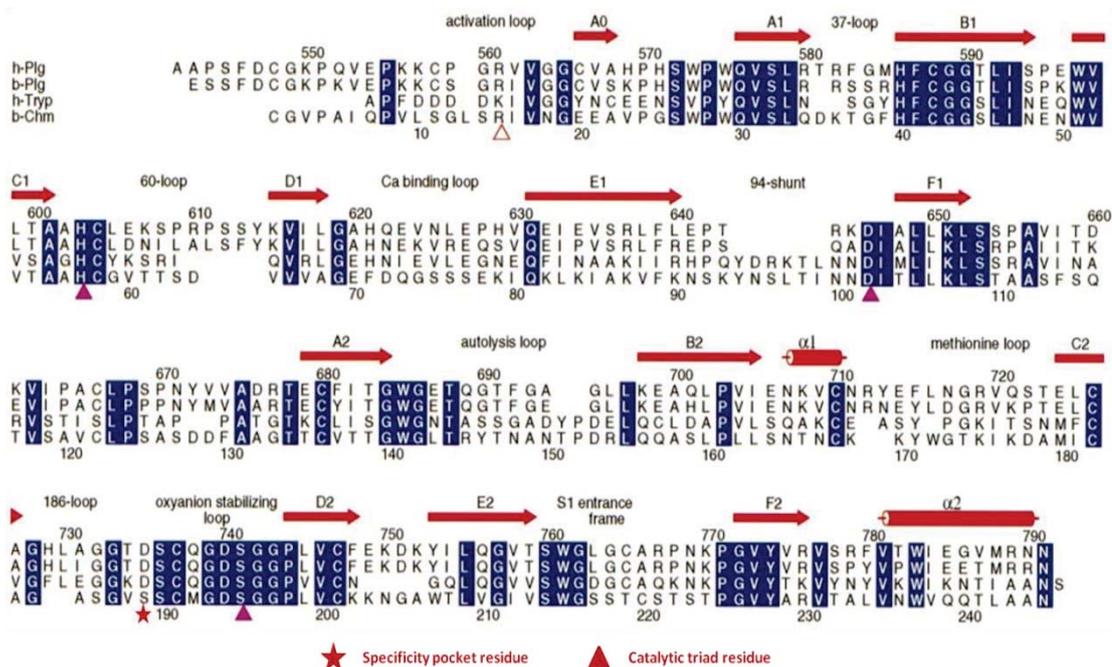


Figure 23. Sequence homology alignment of the catalytic domain: human plasminogen, bovine plasminogen, human trypsin and bovine chymotrypsin. All serine proteases have serine, aspartic acid and histidine in the catalytic triad (represented by the filled triangle) and the residue at the bottom of the specificity pocket (represented by the star) determines the specificity of the protease, e.g. aspartic acid (negatively charged) in the trypsin pocket makes it hydrolyse at lysine or arginine (positively charged) residues. The open triangle indicates the activation cleavage site. Adapted from Wang et al. (2000).

Based on sequence homology with human plasmin and trypsin serine protease (Figure 23) (Wang et al., 2000), bovine plasmin has an Asp730 residue in its substrate-binding pocket, which plays a critical role in determining its specificity towards positively charged lysine and arginine residues on the substrate backbone (Figure 24). The positively charged  $\epsilon$ -amino group of lysine makes a water-mediated salt linkage with the negatively charged carboxylate of the aspartic acid residue and leads to changes in the conformation of the enzyme. The catalytic triad residues become closer and the charge relay transfer begins to make the serine residue of the catalytic triad nucleophilic. Ser736 of the catalytic triad attacks the carboxyl site of the protein chain nucleophilically and leads to hydrolysis.

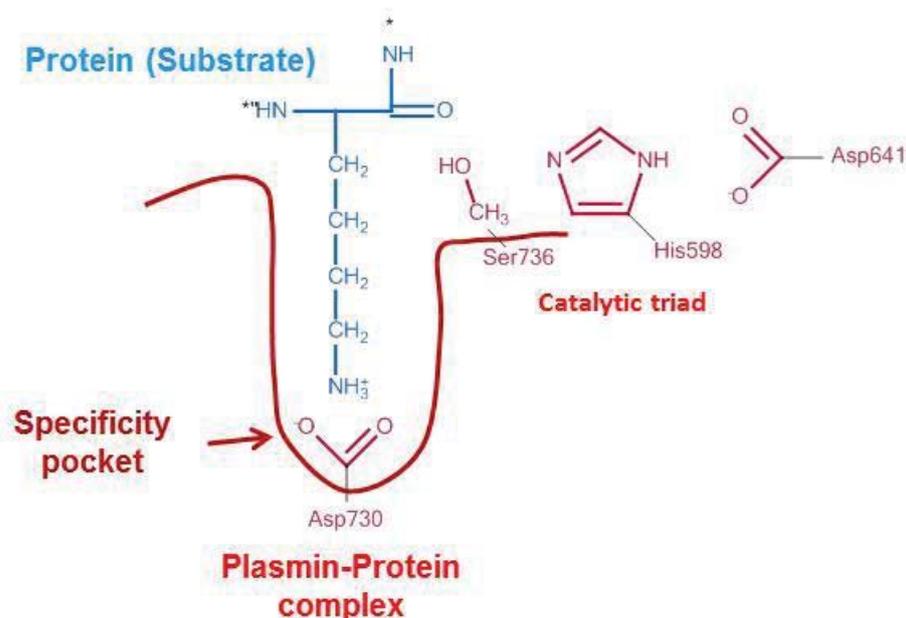


Figure 24. Substrate-binding (specificity) pocket of plasmin, based on sequence homology (Figure 23) and the schematic of trypsin (Figure 22). The charge relay transfer is represented by open arrows and the nucleophilic attack of Ser736 on the substrate is shown by the dashed arrow.

Succinylation causes the succinate molecule to attach to the  $\epsilon$ -amino group of the lysine residue and leads to modification of the charge (replacement of a positive charge with a negative charge) and steric hindrance (attachment of the succinate molecule) (Figure 21). Such modification of the lysine residue of the substrate by succinylation makes it unrecognisable to the substrate-binding pocket (Figure 24). Ultimately, the modified substrate cannot bind to plasmin through the substrate-binding pocket and the carbon ion of the scissile bond cannot be positioned near the nucleophilic serine of the catalytic triad for the hydrolysis, which leads to a reduction in the hydrolysis rate or no hydrolysis.

### 3.6 CONCLUSIONS

The results clearly indicate the negative effect of succinylation on the hydrolysis of  $\beta$ -casein by plasmin. The succinylation of  $\beta$ -casein results in its modification (the  $\epsilon$ -amino group of the lysine residue), making it unrecognisable to the substrate-binding pocket of plasmin; therefore, the substrate is not positioned into the pocket and cannot be hydrolysed by the catalytic triad of plasmin. As confirmed by LC-MS/MS analysis and by identification of succinylation sites, the substrate is modified

mainly by the involvement of the lysine residue in the succinylation reaction, resulting in charge-related and steric-availability-related conformational changes in  $\beta$ -casein.

The present work gives more insight into the complete pattern of the hydrolysis of  $\beta$ -casein by plasmin and shows how the pattern of hydrolysis of succinylated  $\beta$ -casein differs.  $\beta$ -Casein was used as a model system, because it is the primary target for plasmin-induced hydrolysis in milk systems. In the model system,  $\beta$ -casein is not present in the casein micelle, which makes it more prone to succinylation and plasmin-induced hydrolysis.

Further work needs to be done in a real milk system to see the effect of succinylation on plasmin-induced hydrolysis. The proposed mechanism can be used as a basis for developing a plasmin-resistant protein or for controlling plasmin-induced hydrolysis in different ways, e.g. food-grade approaches, by modifying lysine residues of the substrate by transglutaminase cross-linking or by lactosylation.



# Chapter 4: Effect of lactosylation on plasmin-induced hydrolysis of $\beta$ -casein

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## 4.1 INTRODUCTION

The indigenous milk enzyme plasmin (EC3.4.21.7) is an alkaline serine protease (Fox, 1981b) that is similar to trypsin in its activity and characteristics (Chen & Ledford, 1971; Kaminogawa et al., 1972), with an optimum pH of 7.5–8.0 and an optimum temperature of 37°C (Dulley, 1972; Humbert & Alais, 1979). Around 0.14–0.73  $\mu\text{g}$  of plasmin and 0.55–2.75  $\mu\text{g}$  of plasminogen (the zymogen of plasmin) are present in 1 mL of milk (Richardson & Pearce, 1981). The plasmin system is relatively heat stable; it is little affected by the pasteurisation temperature and partially survives ultra-high-temperature (UHT) processing (Bastian & Brown, 1996). Its activity leads to proteolysis in dairy products, e.g. UHT milks and beverages (Chavan et al., 2011), during storage.

Plasmin hydrolyses proteins at the carboxyl site of Lys–X and Arg–X bonds, with a preference for Lys–X bonds (Bastian & Brown, 1996). In milk, plasmin is associated with casein micelles and hydrolyses  $\beta$ -,  $\alpha_{s1}$ - and  $\alpha_{s2}$ -caseins, whereas  $\kappa$ -casein and the whey proteins are resistant to hydrolysis (Ismail & Nielsen, 2010). The caseins contain several lysine and arginine residues, but only a few bonds are hydrolysed rapidly by plasmin. Among the caseins,  $\beta$ -casein is the primary target and is hydrolysed rapidly at its Lys28–Lys29, Lys105–His106 and Lys107–Glu108 bonds. The resulting C-terminal peptides are  $\gamma$ -caseins [ $\gamma_1$ ,  $\beta$ -casein (f29–209);  $\gamma_2$ ,  $\beta$ -casein (f106–209);  $\gamma_3$ ,  $\beta$ -casein (f108–209)] and the corresponding N-terminal peptides are proteose peptones (PP5, PP8slow and PP8fast) (Bastian & Brown, 1996). Other secondary cleavage sites have also been identified (Visser et al., 1989a).

High heat treatment, required in UHT milks and beverages to extend their shelf life, results in several physicochemical changes, such as protein conformational modifications, aggregations and Maillard reactions (van Boekel, 1998). Lactosylation is the first step in the Maillard reaction (Henle, Walter, & Klostermeyer, 1991). It

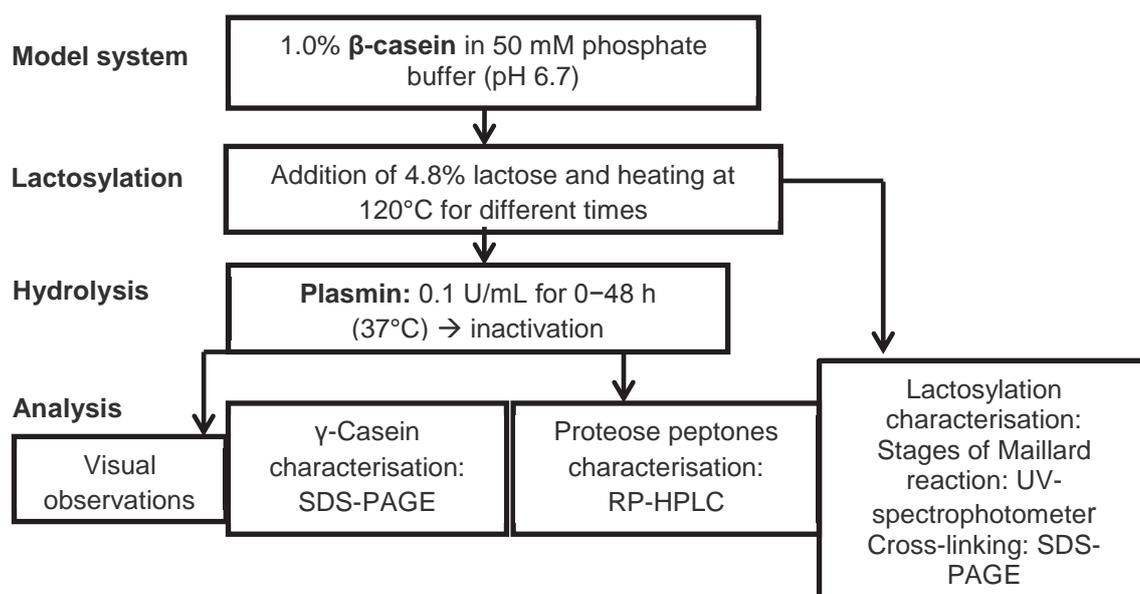
involves lactose and the  $\epsilon$ -amino groups of the lysine residues of milk proteins, which leads to the formation of the stable and dominant intermediate lactulosyl-lysine [the Amadori rearrangement product (ARP)] (Léonil et al., 1997; van Boekel, 1998). Severe heat treatment induces the degradation of lactulosyl-lysine to advanced glycation end-products (Fogliano et al., 1997; Pischetsrieder, Groß, & Schoetter, 1999; van Boekel, 1998). The extent of lactosylation depends on the heat treatments used during processing, the type and structure of the protein (Scaloni et al., 2002; van Boekel, 1998) and the lactose to protein ratio (Evangelisti, Calcagno, & Zunin, 1994). The extent of protein denaturation and glycation is known to increase during the storage of dairy products (Siciliano, Rega, Amoresano, & Pucci, 2000).

According to Scaloni et al. (2002),  $\alpha_{s1}$ -casein and  $\beta$ -casein are the main targets of lactosylation in milk, as they are abundant sources of the accessible  $\epsilon$ -amino groups of lysine residues and are unstructured in milk (Swaisgood, 1992; Venkatachalam, McMahon, & Savello, 1993). Modification of the lysine residue of a substrate affects its accessibility for plasmin hydrolysis, as observed in previous studies on lactosylation (Dalsgaard et al., 2007) and succinylation (Chapter 3). Dalsgaard et al. (2007) studied the proteolysis of milk proteins lactosylated in model systems using a low heat treatment of 65°C for 4 days for lactosylation and observed that the lactosylation of all substrate proteins negatively affected the hydrolysis by plasmin.  $\beta$ -Casein was least affected by lactosylation compared with  $\kappa$ -casein,  $\alpha$ -casein and globular proteins. However, the effects of different degrees of lactosylation, the different stages of the Maillard reaction and the high temperatures that are more relevant to industrial heat treatments have not been studied. Based on previous studies (Dalsgaard et al., 2007; Chapter 3), it can be hypothesised that, with an increase in the degree of lactosylation, the hydrolysis rate should decrease because of the loss of lysine residues; however, involvement of cross-linking and different stages of the Maillard reaction will probably have an impact and make the situation more complex. The objective of the current study was to determine the effect of the degree of lactosylation along with different stages of the Maillard reaction on the inhibition of plasmin-induced hydrolysis in a  $\beta$ -casein model system.

## 4.2 MATERIALS

Pure bovine  $\beta$ -casein [ $> 95\%$  purity, as measured by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)] was made using in-house technology at the Fonterra Research and Development Centre and was used as a substrate. Plasmin from bovine plasma (5 U/mL) was purchased from Roche/Boehringer Mannheim GmbH (Mannheim, Germany). Lactose and chemicals for SDS-PAGE buffers were purchased from Merck (Darmstadt, Germany). An acrylamide/bisacrylamide mixture 37.5:1 (2.6% C) was purchased from Bio-Rad (Hercules, CA, USA). Water-free acetone was purchased from Thermo Fisher Scientific (Auckland, New Zealand). Fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione) was purchased from Sigma-Aldrich (Auckland, New Zealand).

The overall experimental plan is described in Scheme 2.



Scheme 2. Experimental plan.

## **4.3 METHODS**

### **4.3.1 Lactosylation of $\beta$ -casein**

The  $\beta$ -casein model system was prepared by dissolving 1% (w/w)  $\beta$ -casein and 4.8% (w/w) lactose, to match the ratio of  $\beta$ -casein to lactose in milk, in 50 mM sodium phosphate buffer at pH 6.7. Different degrees of lactosylation were achieved by heating the model system in small tightly closed glass vials (4–5 mL) at 120°C for 2, 5, 10 and 20 min in an oil bath, with continuous agitation, followed by immediate cooling by immersion of the vials in ice.

### **4.3.2 Measurement of degree of lactosylation by the fluorescamine method**

The decrease in the number of reactive amino groups on the protein, caused by lactosylation, can be used as a measure of the degree of lactosylation and was measured by the fluorescamine method of O'Connell and de Kruif (2003), as described in Section 3.3.2. All measurements were done in triplicate.

### **4.3.3 Absorbance at 294 nm and 420 nm**

The absorbance and the browning intensity of the samples after heat treatment were measured at room temperature using the method described by Ajandouz, Tchiakpe, Ore, Benajiba, and Puigserver (2001). Using a UV spectrophotometer (Jasco V-560, Jasco Inc., Easton, MD, USA), the absorbance was measured at 294 nm, as an indication of the formation of intermediate products of non-enzymatic browning (Ajandouz et al., 2001), and at 420 nm, as an index of the brown polymers formed in more advanced (final) stages (Hofmann & Frank, 2002; Kato, Matsumura, & Hayase, 1981; Labuza & Saltmarch, 1981; Palombo, Gertler, & Saugy, 1984). The absorbance measurement was carried out after a five-times dilution of the sample in Milli-Q water. All measurements were done in triplicate and mean values were plotted.

### **4.3.4 Plasmin-induced hydrolysis**

The lactosylated model system was hydrolysed by adding plasmin at 15  $\mu$ L/mL (0.1 U/mL) of 1%  $\beta$ -casein. The system was incubated at 37°C, and the hydrolysis pattern was observed at incubation times of 0, 5, 10, 15 and 30 min and 1, 2, 4, 8, 24 and 48

h. Samples were removed after each incubation time and the reaction was stopped immediately by adding 50  $\mu$ L of SDS (2% w/w).

#### **4.3.5 Visual observation**

Changes in the visual appearance of the samples with an increase in the hydrolysis time from the start to 48 h were observed. Samples with different degrees of lactosylation were incubated with plasmin at 37°C and photographs were taken at different times to show the hydrolysis pattern in terms of the increase in turbidity.

#### **4.3.6 SDS-PAGE**

The hydrolysis pattern and the extent of polymerisation of  $\beta$ -casein were studied using 4–15% gradient SDS-PAGE gels (Bio-Rad Laboratories, Auckland, New Zealand), as described in Section 3.3.5.

The decrease in the concentration of monomeric  $\beta$ -casein with an increase in the degree of lactosylation, relative to the control, was measured and characterised as Maillard reaction polymer products. We found that, although the polymerisation of  $\beta$ -casein during the Maillard reaction made quantification difficult, the total volumes of the lanes on the SDS-PAGE gels were observed to be the same for different degrees of lactosylation. For a more thorough understanding, the hydrolysis pattern was analysed by measuring the generation and release of  $\gamma$ -casein ( $\gamma_2$ - +  $\gamma_3$ -casein) and its further hydrolysis.

#### **4.3.7 Reverse-phase high performance liquid chromatography (RP-HPLC)**

The plasmin-induced hydrolysis pattern was analysed by quantifying proteose peptones (hydrophilic portion of  $\beta$ -casein) as described in Section 3.3.6.

All experiments reported were repeated at least twice with the same milk samples. In addition, the experiments were repeated with several different milk samples. Although there were some variations between individual milks, the same trends and relationships as reported here were found for all samples examined to date.

## 4.4 RESULTS AND DISCUSSION

### 4.4.1 Characterisation of lactosylated $\beta$ -casein model system

The lactosylated  $\beta$ -casein model system was characterised by measuring the degree of lactosylation, the stages of the Maillard reaction and the extent of polymerisation. The degree of lactosylation increased linearly ( $R^2 = 0.9981$ ) with an increase in the heating time, indicating a direct correlation (Figure 25). Five different degrees of lactosylation were obtained: control (0), 3.1, 8.5, 20.4 and 40.4%.

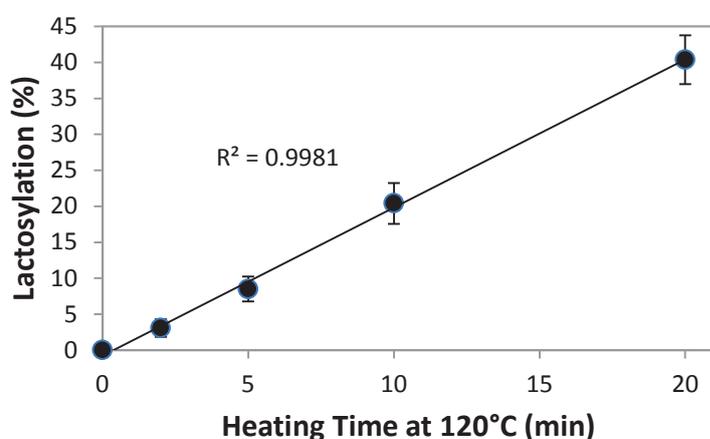


Figure 25. Influence of heating time (0–20 min) at 120°C on the degree of lactosylation in solutions containing 1%  $\beta$ -casein and 4.8% lactose in 50 mM sodium phosphate buffer pH 6.7. Error bars refer to standard deviations.

Various mass spectrometry studies have been done to identify sites of lactosylation in different milk proteins, e.g.  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin,  $\alpha_{S1}$ -casein and  $\beta$ -casein (Arena et al., 2010; Czerwenka, Maier, Pittner, & Lindner, 2006; Fogliano et al., 1998; Léonil et al., 1997; Scaloni et al., 2002; Siciliano et al., 2000). Lactosylation starts with the nucleophilic attack of lactose on the  $\epsilon$ -amino group of lysine on  $\beta$ -casein and leads to the formation of lactulosyl-lysine (ARP) (Fogliano et al., 1998). At relatively low thermal treatment, lactosylation occurs with a degree of specificity in the lactosylation sites (Fogliano et al., 1998). Such attachment of lactose molecules to the lysine residues of  $\beta$ -casein occurs, especially at Lys107 (under mild conditions) and Lys32, Lys48, Lys107, Lys113 and Lys176 (under intensive heat treatment) (Scaloni et al., 2002). Henle and Klostermeyer (1993) found Lys28/29,

Lys32, Lys99, Lys107 as high, Lys105, Lys113, Lys176 as moderate and Lys48, Lys97, Lys169 as low reactivity sites of  $\beta$ -casein A1 for the Maillard reaction.

The absorbance at the five different degrees of lactosylation was measured at 294 nm and 420 nm to detect the formation of intermediate and advanced-stage products respectively (Figure 26). The formation of intermediate products of the Maillard reaction (uncoloured polymer compounds) was in agreement with observations of Ajandouz et al. (2001) and was found to increase with an increase in the degree of lactosylation (Figure 26).

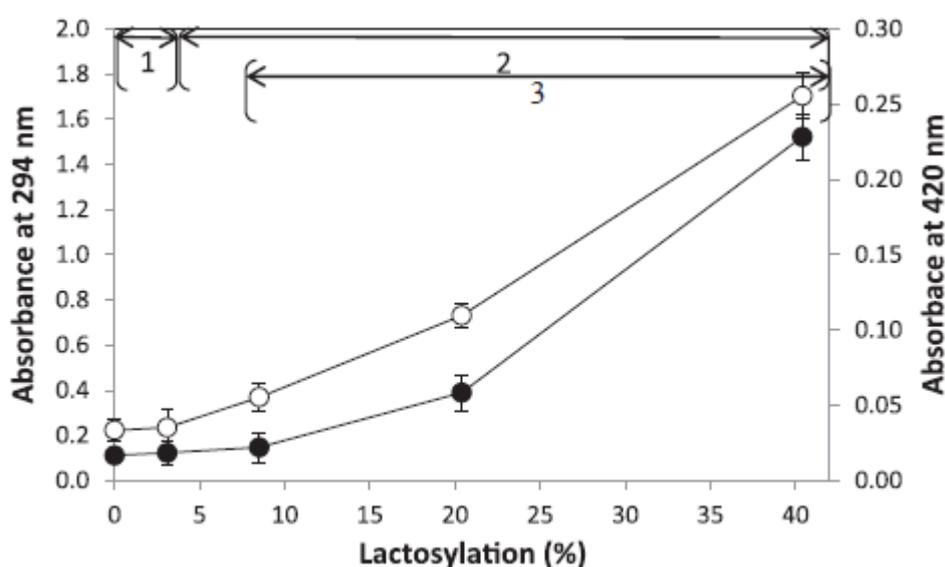


Figure 26. Characterisation of stages of the Maillard reaction in a  $\beta$ -casein model system. Effect of lactosylation level on UV absorbance measurement at 294 nm (indicator of the formation of polymers – intermediate stage) and at 420 nm (browning): 1, initial stage; 2, intermediate stage (polymerisation); 3, advanced stage (browning). Error bars refer to standard deviations.

The intermediate products formed mainly beyond 3.1% lactosylation and could also be correlated with the SDS-PAGE results, which followed an identical pattern of a linear increase in polymerisation beyond 3.1% lactosylation (Figure 27). According to Lericci, Barbanti, Manzano, and Cherubin (1990), these uncoloured polymer compounds are the precursor of the Maillard reaction. The formation of such intermediate products could decrease the content of available lysine.

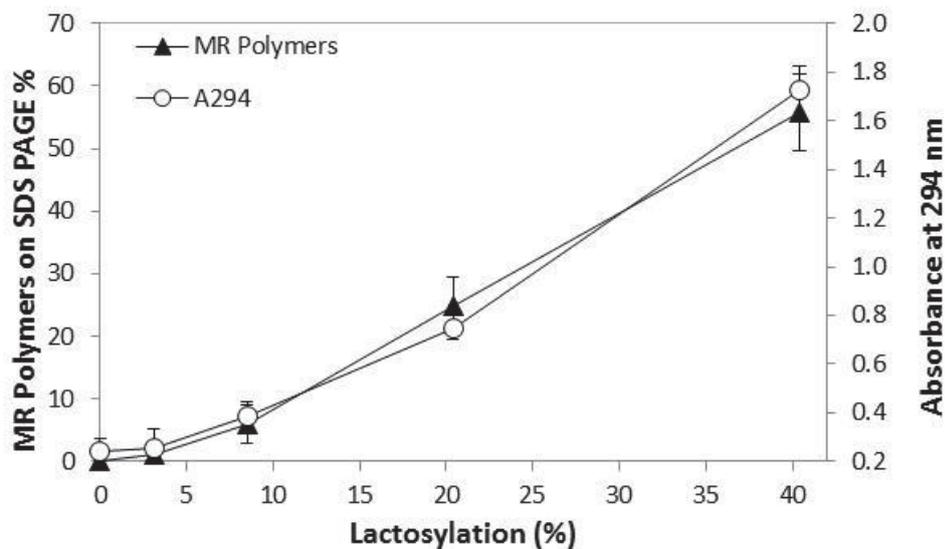


Figure 27. Effect of lactosylation on Maillard reaction (MR) polymers (cross-linked), as measured using SDS-PAGE, and the absorbance at 294 nm (A294). Error bars refer to standard deviations.

The absorbance at 420 nm increased more rapidly (Figure 26) beyond 8.5% lactosylation, indicating the formation of product from the advanced stage of the Maillard reaction, which further decreased the availability of lysine for different reactions. This was mainly because, beyond 8.5% lactosylation, extensive heat treatment of the intermediate stage product resulted in the formation of mainly unsaturated, brown nitrogenous polymers and copolymers (Ames, 1990; Hodge, 1953) known as advanced glycation end-products (Friedman, 1996; van Boekel, 1998), which were identified as cross-linked polymers (Figure 27) using SDS-PAGE. Maillard-reaction-induced cross-linking was also observed in previous studies on fructose–lysine model systems (Ajandouz et al., 2001),  $\beta$ -casein model systems (Pellegrino, van Boekel, Gruppen, Resmini, & Pagani, 1999) and stored UHT milks (Al-Saadi, Easa, & Deeth, 2013; Andrews & Cheeseman, 1972).

#### 4.4.2 Effect of degree of lactosylation on plasmin-induced hydrolysis of $\beta$ -casein

The loss of lysine residues and cross-linking during different stages of the Maillard reaction could affect the plasmin-induced hydrolysis of  $\beta$ -casein and the release and further hydrolysis of hydrolysed peptides, e.g.  $\gamma$ -caseins (hydrophobic peptides) and proteose peptones (hydrophilic peptides). As hydrophobic and hydrophilic peptides might be lactosylated differently, which could have an effect on their hydrolysis,

SDS-PAGE and RP-HPLC respectively, as well as visual changes, were used to analyse  $\gamma$ -casein and proteose peptones to gain detailed insight into this effect.

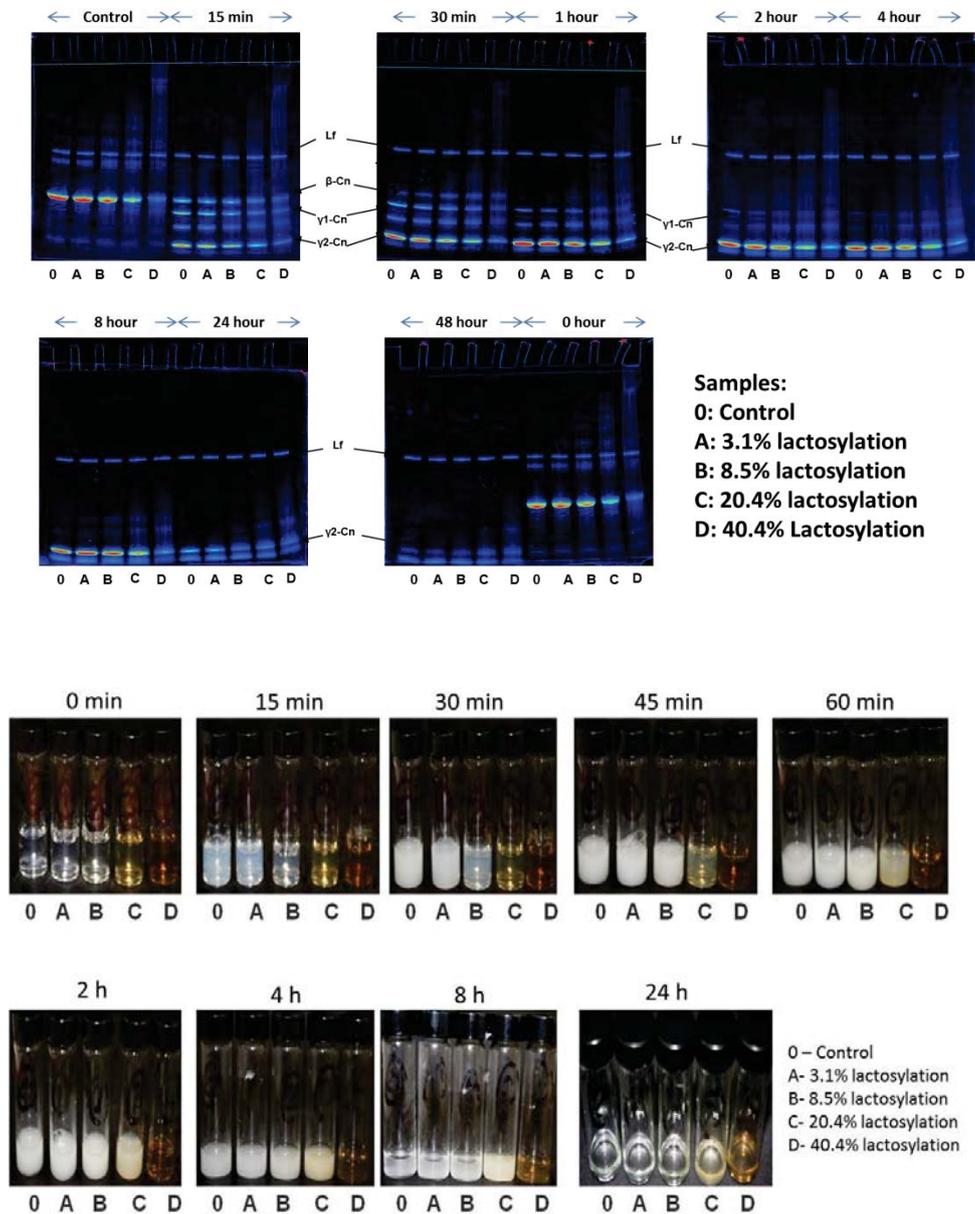


Figure 28. Plasmin-induced hydrolysis of lactosylated  $\beta$ -casein. The 1%  $\beta$ -casein model system was lactosylated to different degrees (0, 3.1, 8.5, 20.4 and 40.4%) and then hydrolysed by plasmin for up to 48 h. A. SDS page images showing hydrolysis of  $\beta$ -casein and generation of  $\gamma$ -casein in initial stages and hydrolysis of  $\gamma$ -casein in the later stages of the hydrolysis. B. Visual observations were made by taking samples at 0 min (before plasmin addition), and 15, 30, 45 and 60 min and 2, 4, 8 and 24 h after plasmin addition.

The plasmin-induced hydrolysis of  $\beta$ -casein leads to a change in turbidity that can be observed visually (Figure 28). The control sample (non-lactosylated) and that with a low degree of lactosylation (3.1%) became turbid within the first 15 min of the hydrolysis reaction, whereas those with greater degrees of lactosylation (20.4 and 40.4%) were still clear after 30 min. The turbidity, which is dependent on the degree of lactosylation, increased with an increase in the hydrolysis time until it reached a maximum, and then decreased following the same order as the increase, with the samples becoming transparent again after 24 h (Figure 28).

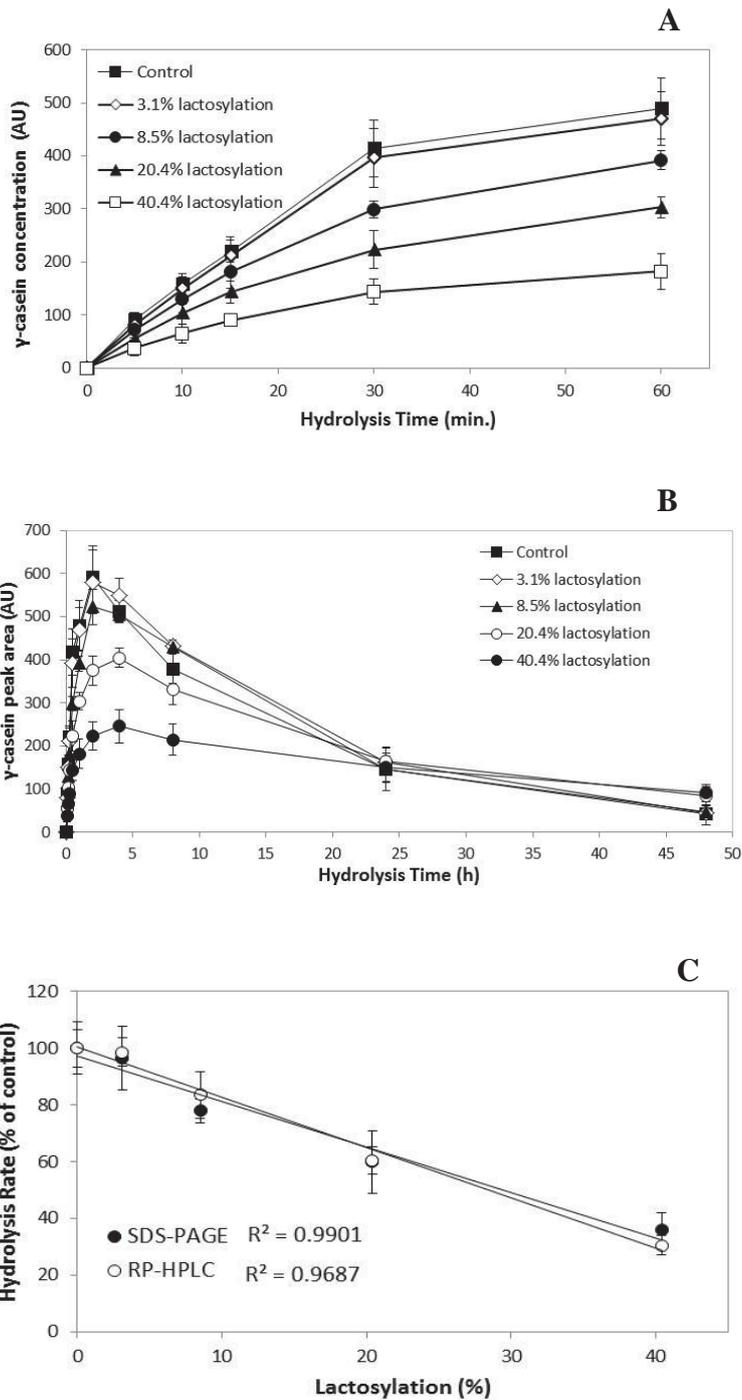


Figure 29. Effect of degree of lactosylation of  $\beta$ -casein on its hydrolysis by plasmin. A.  $\gamma$ -Casein formation during 48 h of hydrolysis was quantified using SDS-PAGE. B. Effect of the degree of lactosylation on the rate of plasmin-induced hydrolysis. The hydrolysis rate was calculated as a percentage of the control from the first 60 min of the hydrolysis pattern. Error bars refer to standard deviations.

The increase in turbidity correlated well with the increase in the concentration of  $\gamma$ -casein (Figure 29) and it was hypothesised that the increase in turbidity was due

mainly to the release of hydrophobic peptides, i.e.  $\gamma$ -caseins (especially  $\gamma_2$ - and  $\gamma_3$ -caseins), during plasmin-induced hydrolysis (as explained in Figure 30). The  $\beta$ -caseins in the model system are associative, forming simple micelles consisting of a single type of protein at room temperature (de Kruif & Grinberg, 2002). The hydrophobic portion (the C terminus of  $\beta$ -casein) forms the core of the  $\beta$ -casein micelle and the hydrophilic portion (the N terminus) protrudes from the micelle (Aschi, Calmettes, Daoud, Douillard, & Gharbi, 2009; de Kruif & Grinberg, 2002; O’Connell, Grinberg, & de Kruif, 2003). In this micellar system, the sites targeted by plasmin, i.e. Lys105, Lys107 and Lys28/29, were easily accessible and were readily hydrolysed to  $\gamma$ -casein (hydrophobic portion) and proteose peptones (hydrophilic portion); the hydrophobic peptides aggregated via hydrophobic interactions (Figure 30), resulting in an increase in the turbidity of the solutions (Figure 28). The generation and/or release of hydrophobic peptides by plasmin-induced hydrolysis decreased with an increase in the degree of lactosylation, which was clearly seen by reduced or no turbidity in lactosylated samples upon plasmin-induced hydrolysis.

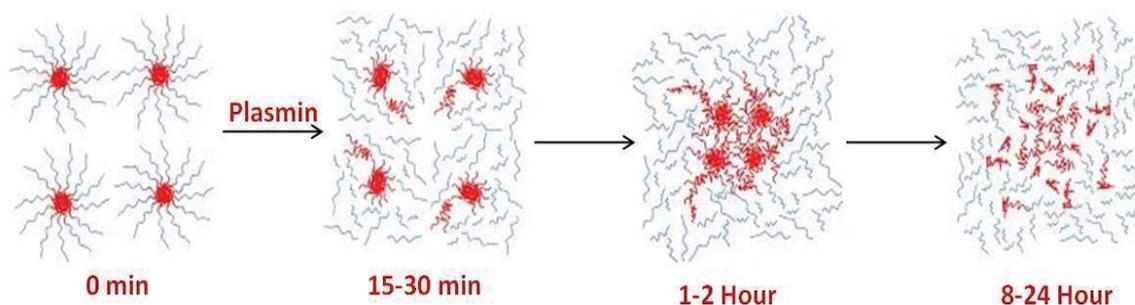


Figure 30. Schematic representation of plasmin-induced hydrolysis of a  $\beta$ -casein model system (red,  $\gamma$ -caseins; blue, proteose peptones).

The hydrolysis reaction was analysed by monitoring  $\gamma$ -casein ( $\gamma_2$ - and  $\gamma_3$ -caseins) and proteose peptones. Lactosylation of  $\beta$ -casein was found to affect plasmin-induced hydrolysis (Figure 29A, Figure 29B and Figure 31). An increase in the degree of lactosylation negatively affected the generation of  $\gamma$ -casein (Figure 29) and proteose peptones, e.g. PP5, PP8slow and PP8fast (Figure 31), by plasmin-induced hydrolysis throughout the hydrolysis time up to 48 h. The initial hydrolysis rate was assessed from the tangent of the first 1 h of the hydrolysis reaction. The RP-HPLC results (Figure 31) were in agreement with the SDS-PAGE results (Figure 29). There was a

marked reduction in the hydrolysis of  $\beta$ -casein at greater degrees of lactosylation, and the reduction in the rate of the reaction was linear (Figure 29B). The peak  $\gamma$ -casein concentration was used as an indicator of the maximum amount of substrate being hydrolysed by plasmin. The peak  $\gamma$ -casein concentration was less in lactosylated samples than in the control (Figure 29A). Analysis of the secondary stage of the hydrolysis ( $\gamma$ -casein hydrolysis), when  $\beta$ -casein was absent and  $\gamma$ -casein was the main substrate for hydrolysis, also suggested that lactosylation had a negative effect on the plasmin-induced hydrolysis of  $\gamma$ -casein.

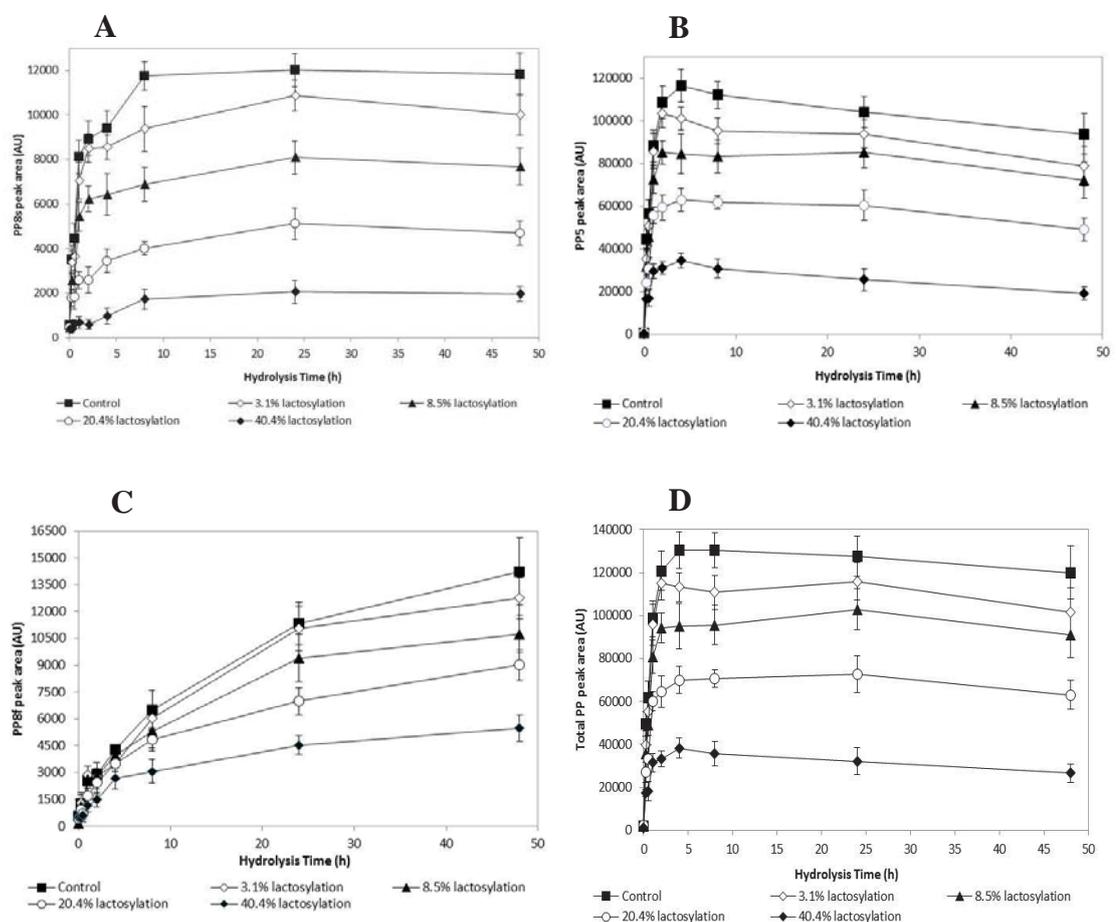


Figure 31. Comparative hydrolysis patterns of the complete plasmin-induced hydrolysis of  $\beta$ -casein, as quantified using RP-HPLC: A, PP8slow; B, PP5; C, PP8fast; D, total proteose peptones. The areas under the peaks for different peptides were calculated and are presented as an increase in the peptide concentration (AU – arbitrary units). Note the change in scale. Error bars refer to standard deviations.

During lactosylation, the formation of lactulosyl-lysine caused a linear loss of lysine residues in the early stages of the reaction and the same or other lysine residues became involved in Maillard-induced cross-linking as the reaction advanced; however, the loss of lysine residues was still linear, as measured by the fluorescamine method. As both the decrease in free lysine on heat treatment and the decrease in hydrolysis with lactosylation were linear, it was concluded that the loss of lysine was the main reason for the decrease in hydrolysis, independent of the stages of the Maillard reaction.

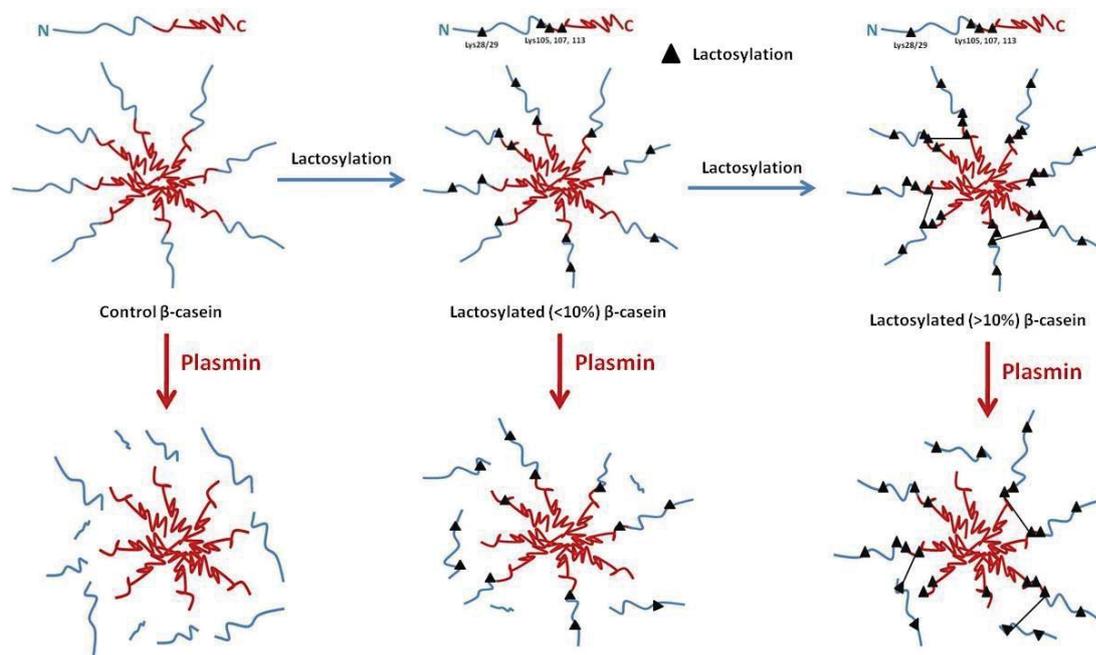


Figure 32. Resistance of lactosylated  $\beta$ -casein to plasmin-induced hydrolysis. Lactosylation makes protein unrecognisable to plasmin through lysine capping and, at greater degrees of lactosylation, cross-linking also affects the release of hydrolysed peptides.

Plasmin hydrolyses after Lys28, Lys105 and Lys107 of the  $\beta$ -casein system; however, because of lactosylation of these lysine residues, e.g. Lys28/29 (Henle & Klostermeyer, 1993), Lys107 (Scaloni et al., 2002) and Lys105 (Arena et al., 2010), there was a decrease in the primary stage of plasmin-induced hydrolysis. As reactions continued, the hydrolysis of  $\gamma$ -casein was also affected, which could also have been due to lactosylation at the Lys107, Lys113, Lys169 and Lys176 residues of  $\beta$ -casein (Arena et al., 2010; Scaloni et al., 2002), making it resistant to further hydrolysis by plasmin. Some of the lactosylated peptides identified using RP-HPLC–

MS are shown in Appendix 6. Attachment of the lactose molecule to the  $\epsilon$ -amino group of a lysine residue leads to charge modification (the removal of positive charge) and steric hindrance (attachment of the large lactose molecule). Such modification of the lysine residue of the substrate by lactosylation makes it unrecognisable to the substrate specificity (binding) pocket (as explained by Figure 24). Ultimately, the modified substrate cannot bind to plasmin through the substrate-binding pocket and the carbon ion of the scissile bond cannot be positioned near the nucleophilic serine of the catalytic triad for hydrolysis, which leads to a reduction in hydrolysis or no hydrolysis. In advanced stages of the Maillard reaction, Maillard-induced cross-linking may also play an important role in the decreased release of hydrolysed peptides from the protein; it is important to note that it is the lactosylated residues that are involved in the cross-linking reaction. Therefore, the combined effect, involving lactosylated lysine residues in the Maillard reaction, i.e. the decrease in substrate availability and the decrease in the release of hydrolysed peptides, is responsible for the decrease in plasmin-induced hydrolysis (as shown in Figure 32).

#### **4.5 CONCLUSIONS**

The present study demonstrates the inhibitive effect of lactosylation on the hydrolysis of  $\beta$ -casein by plasmin. Lactosylation of  $\beta$ -casein results in substrate modification (the  $\epsilon$ -amino groups of lysine residues), making casein unrecognisable to the substrate-binding pocket of plasmin; therefore, the substrate is not positioned into the pocket and cannot be hydrolysed by the catalytic triad of plasmin and, in advanced stages of the Maillard reaction, cross-linking affects the release of hydrolysed peptides. Modification of the substrate is mainly by involvement of the lysine residue in the lactosylation reaction, resulting in the formation of lactulosyl-lysine (the ARP) and conformational changes in  $\beta$ -casein. The present work gives further insight into the complete hydrolysis pattern of  $\beta$ -casein, indicating a different hydrolysis pattern in a system with a modified substrate, e.g. a model system lactosylated to different degrees. This work gives a clear indication of the degree of lactosylation required to inhibit plasmin-induced hydrolysis, and can be applied to balance both plasmin-induced hydrolysis and the degree of

lactosylation in the real milk system.  $\beta$ -Casein was used as a model system in the present work, as it is the primary target of plasmin-induced hydrolysis in milk systems. However, we need to consider that the  $\beta$ -casein in the model system was not part of the casein micelle, which might make it more prone to both lactosylation and plasmin-induced hydrolysis. Therefore, further work is required to determine the effect of lactosylation on plasmin-induced hydrolysis in a real system. The proposed mechanism can be used as a tool to develop a plasmin-resistant protein or to control plasmin-induced hydrolysis by different methods of substrate modification.

# Chapter 5: Effect of transglutaminase-induced cross-linking on plasmin-induced hydrolysis

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## 5.1 INTRODUCTION

Milk protein modifications are widely used for the improvement of functional properties. Transglutaminase (TG)-induced modification of food proteins improves different functional properties such as emulsifying capacity, gelation, solubility, foaming capacity and stability, lysine protection from various chemical reactions, as an aid in encapsulation of lipids and/or lipid-soluble material, elasticity and water-holding capacity (Motoki, Nio, & Takinami, 1984; Özrenk, 2006; Yildirim, Hettiarachchy, & Kalapathy, 1996). Many people have comprehensively reviewed the application of TGs in the cross-linking of food proteins (De Jong & Koppelman, 2006; Ha & Iuchi, 2002; Jaros, Partschefeld, Henle, & Rohm, 2006; Motoki & Seguro, 1998; Özrenk, 2006; Yokoyama, Nio, & Kikuchi, 2004).

Transglutaminase (TG; protein-glutamine  $\gamma$ -glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyses a post-translational modification of proteins by an acyl group transfer between  $\gamma$ -carboxamide groups of peptide-bound glutamine residues (acyl donor) and the primary amino groups in a variety of amine compounds (acyl acceptor), including peptide-bound  $\epsilon$ -amino groups of lysine residues, and results in the formation of inter- or intramolecular covalent cross-links between glutamine and lysine residues in proteins (De Jong & Koppelman, 2006; Jaros et al., 2006; Lorenzen, Schlimme, & Roos, 1998; Özrenk, 2006; Yokoyama et al., 2004).

In milk and dairy protein systems, plasmin-induced proteolysis is a major concern, limiting their shelf life through the release of hydrolysed peptides. These peptides cause flavour (bitterness) and texture (gelation and sedimentation) defects (Bastian & Brown, 1996). Therefore, it is important to control plasmin-induced proteolysis. Plasmin hydrolyses proteins on the carboxyl site of Lys-X and Arg-X bonds, with a preference for the Lys-X bond (Bastian & Brown, 1996).

Lysine residues on the  $\beta$ -casein backbone were cross-linked with glutamyl residues to different extents using TG. In previous studies, succinylation (Chapter 3) and lactosylation (Chapter 4) were found to be useful in controlling plasmin-induced hydrolysis; however, the mechanism became complicated in the lactosylation reaction, in which cross-linking was also involved. Therefore, the aim of the present work was to explore a food-grade modification approach to substrate modification, involving cross-linking to understand the effect of both lysine modification and cross-linking on the plasmin-induced hydrolysis of  $\beta$ -casein (the primary target of plasmin) and to compare it with succinylation and lactosylation.

## 5.2 MATERIALS

Pure bovine  $\beta$ -casein [ $> 95\%$  purity, as measured by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)] was made using in-house technology at the Fonterra Research and Development Centre and was used as a substrate. Plasmin from bovine plasma (5 U/mL) was purchased from Roche/Boehringer Mannheim GmbH (Germany). TG ( $\text{Ca}^{+2}$ -independent ACTIVA-WM) derived from the microorganism *Streptoverticillium* was supplied by Ajinomoto Co. Inc. (Japan). Ajinomoto TG comes in a powdered form and has 1% enzyme concentration and 99% maltodextrin. Chemicals for SDS-PAGE buffers were purchased from Merck (Darmstadt, Germany). An acrylamide/bisacrylamide mixture 37.5:1 (2.6% C) was purchased from Bio-Rad (Hercules, CA, USA). Water-free acetone was purchased from Thermo Fisher Scientific (Auckland, New Zealand). Fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione) was purchased from Sigma–Aldrich (Auckland, New Zealand).

## 5.3 METHODS

### 5.3.1 Transglutamination of $\beta$ -casein

The  $\beta$ -casein model system was prepared by dissolving 1%  $\beta$ -casein in 50 mM phosphate buffer at pH 6.7. TG-induced cross-linking was achieved by the addition of Ajinomoto TG at 1 mg/50 mg of protein to the pre-incubated  $\beta$ -casein model system at 37°C and different extents of cross-linking were achieved by varying the incubation time – 0, 5, 10, 30 and 60 min. The TG was inactivated by heating the system at 75°C for 5 min. The extent of cross-linking was measured using SDS-PAGE.

### 5.3.2 Plasmin-induced hydrolysis

The TG-induced cross-linked  $\beta$ -casein model system was hydrolysed by plasmin added at 15  $\mu\text{L}/\text{mL}$  (0.1 U/mL) of the  $\beta$ -casein model system, as described in Section 3.3.4.

### **5.3.3 SDS-PAGE**

To study the effect of transglutamination on proteins and the hydrolysis pattern of proteins, SDS-PAGE was performed using the method of Anema and Klostermeyer (1997), as described in Section 3.3.5.

### **5.3.4 Reverse-phase high performance liquid chromatography (RP-HPLC)**

pH-4.6-soluble fractions were prepared and analysed using RP-HPLC, using the method described in Section 3.3.6.

All experiments reported were repeated at least twice with the same milk samples. In addition, the experiments were repeated with several different milk samples. Although there were some variations between individual milks, the same trends and relationships as reported here were found for all samples examined to date.

## 5.4 RESULTS AND DISCUSSION

### 5.4.1 Extent of TG-induced cross-linking

The  $\beta$ -casein model system was cross-linked by TG to achieve different extents of cross-linking. The extent of cross-linking by transglutamination was measured using SDS-PAGE (Table 5, Figure 33).

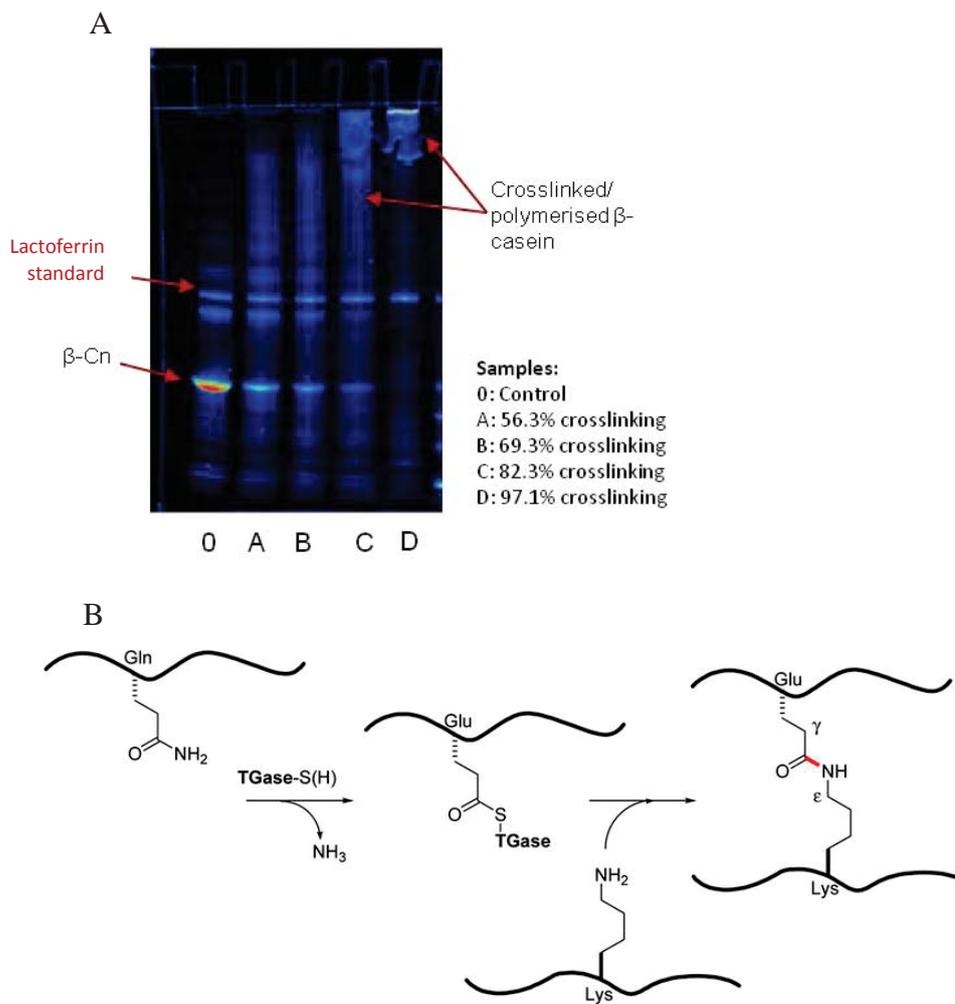


Figure 33. A. TG-induced cross-linking of  $\beta$ -casein. SDS-PAGE was used to measure the extent of cross-linking, the single bands of  $\beta$ -casein on SDS-PAGE were quantified and the decrease in the monomeric band due to cross-linking was noted. B. Typical transglutaminase-induced cross-linking reaction (De Jong & Koppelman, 2006).

Gradient (4–15%) SDS-PAGE was used to observe the clear increase in cross-linking/polymerisation of  $\beta$ -casein. As the reaction time for transglutamination increased, a decrease in the monomeric  $\beta$ -casein band and a smearing or trailing effect were observed, because of the formation of multiple species of different

molecular weights by TG-induced cross-linking (Figure 33). The decrease in the monomeric  $\beta$ -casein band was quantified and was represented as the percentage of TG-induced cross-linking (Table 5).

In the milk system, caseins are favourable substrates for TG-induced cross-linking because their open tertiary structure facilitates lysine and glutamine availability, in contrast to globular whey proteins (Kurth & Rogers, 1984; Singh, 1991). Among the caseins,  $\beta$ -casein and  $\kappa$ -casein have higher reactivity than  $\alpha$ -casein for TG reactions, which can be attributed to the difference in content and availability of glutamyl and lysine residues and the type of TG and its specificity towards glutamyl residues (De Jong & Koppelman, 2006; Huppertz & de Kruif, 2007; Traoré & Meunier, 1991).

Table 5. Extent of TG-induced cross-linking measured as a decrease in the monomeric  $\beta$ -casein band on SDS-PAGE and expressed as cross-linking (%)

Sample name	Incubation time (min)	$\beta$ -Casein (SDS-PAGE AU)	Cross-linking (%)
O	0	42799154	0.0
A	5	18695354	56.3
B	10	13135702	69.3
C	30	7586702	82.3
D	60	1255310	97.1

In the present study, the  $\beta$ -casein model system was cross-linked by TG at 37°C, which should facilitate intermolecular cross-linking of  $\beta$ -casein, as described earlier by O'Connell and de Kruif (2003). The treatment of  $\beta$ -casein with TG leads to intermolecular cross-linking at higher temperature ( $\sim 35^\circ\text{C}$ ), but leads to intramolecular cross-linking at lower temperature ( $\sim 0^\circ\text{C}$ ), which inhibits micellisation by reducing molecular mobility and/or causes an alteration in the charge distribution of  $\beta$ -casein.

#### 5.4.2 Effect of TG-induced cross-linking on plasmin-induced hydrolysis

The samples with different extents of cross-linking were hydrolysed by plasmin for 0–48 h under the same conditions and the hydrolysed samples were analysed in depth using SDS-PAGE and RP-HPLC, as described in Sections 3.3.5 and 3.3.6.

As well as a decrease in the  $\beta$ -casein band, a decrease in the cross-linked/polymerised  $\beta$ -casein (the smearing tail) on SDS-PAGE was also observed with an increase in the hydrolysis time (Figure 34), suggesting that the cross-linked  $\beta$ -casein is accessible and is hydrolysed by plasmin. The primary stage of the hydrolysis was found to be affected negatively, as observed in Figure 35; with an increase in the TG-induced cross-linking, the amount of  $\gamma$ -casein released decreased.

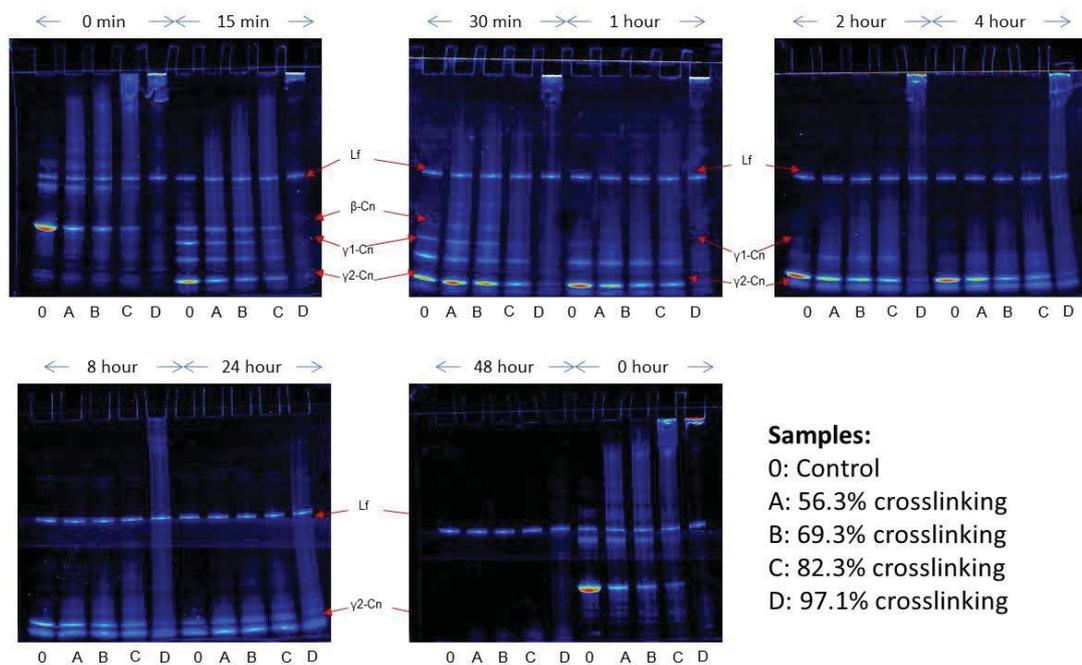


Figure 34. Typical SDS-PAGE gels for the plasmin-induced hydrolysis analysis:  $\beta$ -casein samples with different extents of cross-linking were hydrolysed by plasmin and analysed by quantification of the  $\gamma$ -casein using gradient (4–15%) SDS-PAGE. Amido Black was used for staining and quantification was carried out using ImageQuant software. Samples were analysed at 0 min (control – samples with no hydrolysis), and after hydrolysis for 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and 48 h. Lf: lactoferrin was used as a standard.

There was only a 12.9% decrease in the hydrolysis rate with 56.3% cross-linking; this suggested that a low extent of TG-induced cross-linking did not have much effect on the plasmin-induced hydrolysis or the release of  $\gamma$ -caseins. As the extent of TG-induced cross-linking was increased from 56.3 to 69.3%, the decrease in the hydrolysis rate became 28.2%. Further increases in the extent of TG-induced cross-linking had a marked effect on the hydrolysis rate; with 82.3 and 97.1% cross-

linking, the decrease in the hydrolysis rate became 56.9 and 90.4% respectively. The decrease in the hydrolysis rate followed a linear trend ( $R^2 = 0.9825$ ) beyond 56.3% TG-induced cross-linking (Figure 35B), suggesting that, whereas a low level of cross-linking did not have much effect on the hydrolysis rate, the decrease in hydrolysis became linear at > 50% cross-linking.

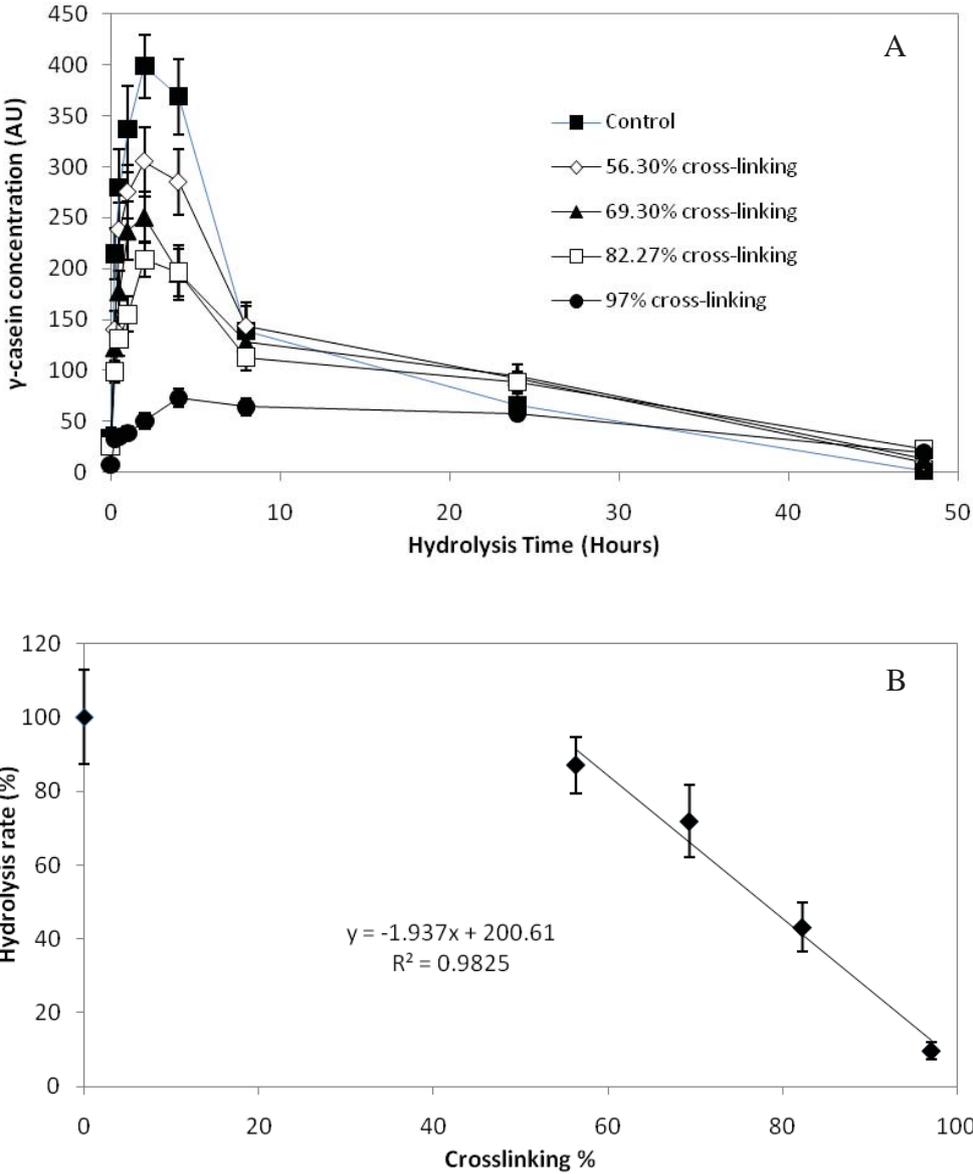


Figure 35. Effect of TG-induced cross-linking on plasmin-induced hydrolysis of  $\beta$ -casein. A. Complete hydrolysis pattern plotted by quantification of  $\gamma$ -casein on SDS-PAGE. The complete reaction was observed for 48 h to achieve the end-point when no visible peptide band remained on SDS-PAGE. B. Effect of TG-induced cross-linking on plasmin-induced hydrolysis rate. Error bars refer to standard deviations.

The control samples had maximum peak values and the peak value decreased with an increase in the extent of cross-linking (Figure 35A). The  $\gamma$ -casein peak value did not shift with an increase in cross-linking (Figure 35A), which was in contrast to the shift observed in succinylated and lactosylated samples. The decrease in the peak value due to cross-linking indicated the negative effect on the hydrolysis rate or less hydrolysis of  $\beta$ -casein.

RP-HPLC was helpful for thorough analysis of the plasmin-induced hydrolysis pattern. N-terminal peptides, i.e. PP5, PP8slow and PP8fast, were clearly identified and quantified and the formation of multiple proteose peptone species due to cross-linking was also characterised (Figure 36).

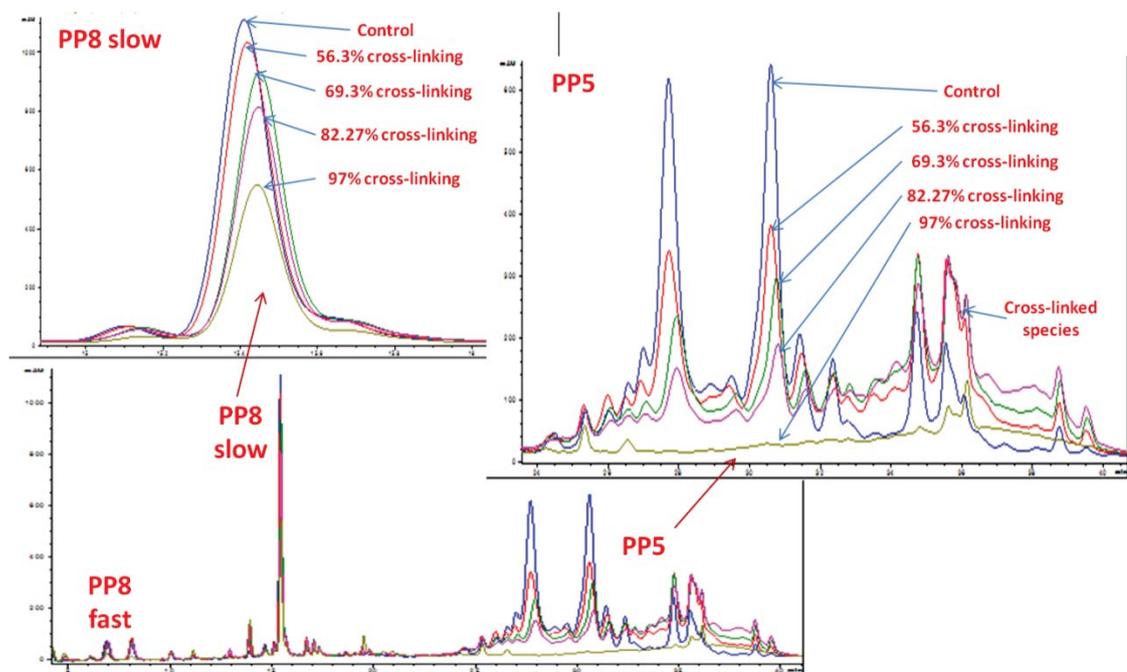


Figure 36. Comparative hydrolysis patterns observed on RP-HPLC (overlays) after 2 h of hydrolysis.

TG-induced cross-linking negatively affected the plasmin-induced hydrolysis, as observed using RP-HPLC and in agreement with the SDS-PAGE results. With an increase in cross-linking, the elution patterns of the peptide peaks changed and the peaks became smaller because there was less peptide formation or less release as a result of the cross-linking (Figure 36). In the primary stage of the hydrolysis, the patterns for PP8slow and PP8fast were different from those for PP5 and total proteose peptones. The increase in PP8fast was not clear (Figure 37), which could

have been due to its generation and then its rapid hydrolysis into smaller peptides; in contrast, PP8slow and PP5 showed clear patterns of an increase in concentration with an increase in the hydrolysis time. The pattern for PP8slow suggested that cross-linking did not have much effect on its generation; the control and all samples except the 97.1% cross-linked sample had identical hydrolysis patterns (Figure 37A). The effect of TG-induced cross-linking was clearly seen for PP5; the PP5 concentration decreased with an increase in cross-linking (Figure 37B), suggesting either less release of PP5 or less hydrolysis of  $\beta$ -casein or both. The effect of TG-induced cross-linking on the total proteose peptones was quite similar to the effect on PP5, which was mainly due to the prominence of PP5 in the total concentration of proteose peptones (Figure 37D).

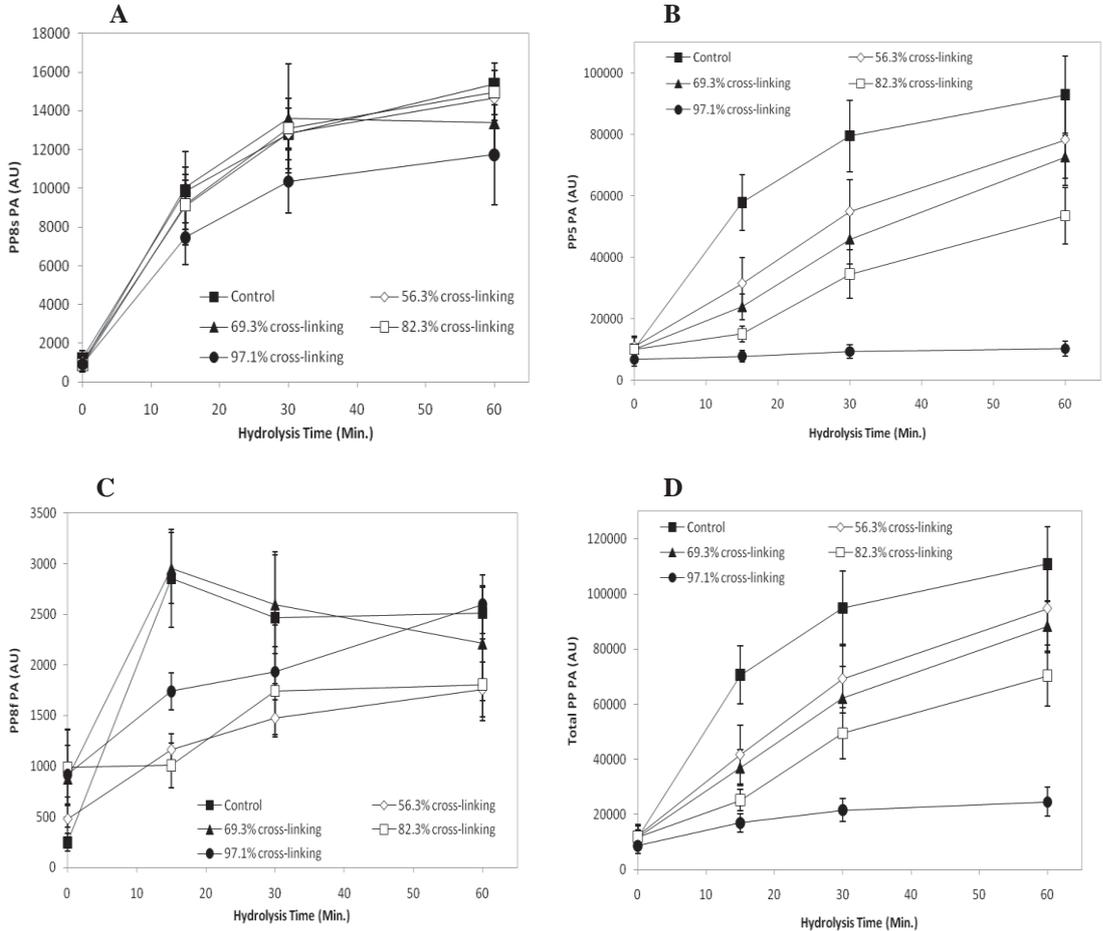


Figure 37. Comparative hydrolysis patterns for the initial hydrolysis: A, PP8slow; B, PP5; C, PP8fast; D, total proteose peptones. Areas under the peaks for the different peptides were calculated and presented as an increase in the peptide concentration. Error bars refer to standard deviations. Note scale differences.

The release of PP8slow was not much affected, which could have been because fewer residues were involved in cross-linking; in contrast, the release of PP5 was significantly affected, suggesting the involvement of PP5 in cross-linking and that this involvement might not have affected its further hydrolysis into PP8slow and PP8fast in the primary stage of hydrolysis (Figure 37).

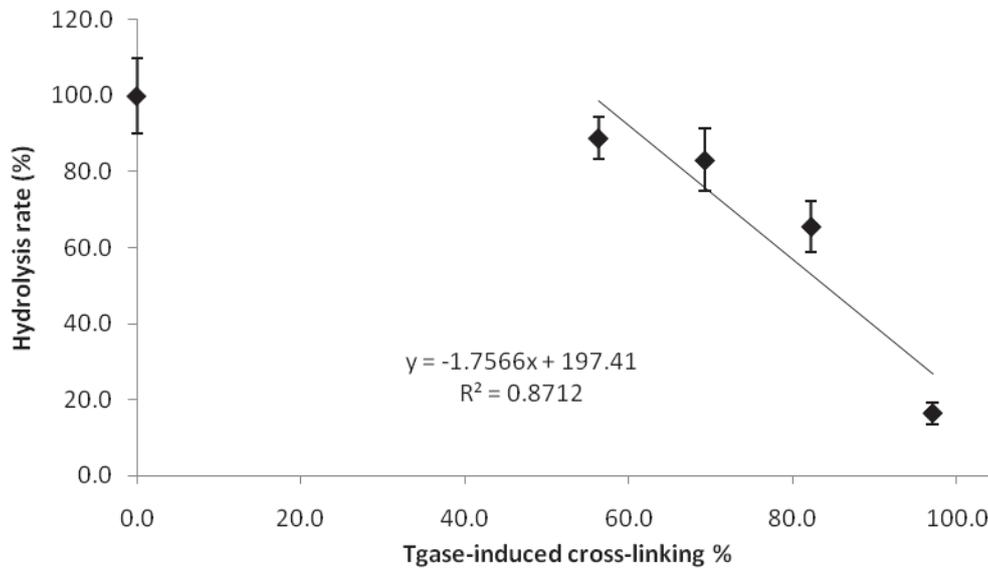


Figure 38. Effect of TG-induced cross-linking on plasmin-induced hydrolysis of  $\beta$ -casein, measured as the generation of total proteose peptones during the initial stage of the hydrolysis. Error bars refer to standard deviations.

The rate of plasmin-induced hydrolysis of  $\beta$ -casein was not much affected at low extents of cross-linking. For example, at 56.3% cross-linking, the rate decreased by only 11.2%. However, at higher extents of cross-linking, the rate decreased markedly. Beyond 56.3% cross-linking, the plasmin-induced hydrolysis rate decreased linearly ( $R^2 = 0.8712$ ) with an increase in TG-induced cross-linking (Figure 38).

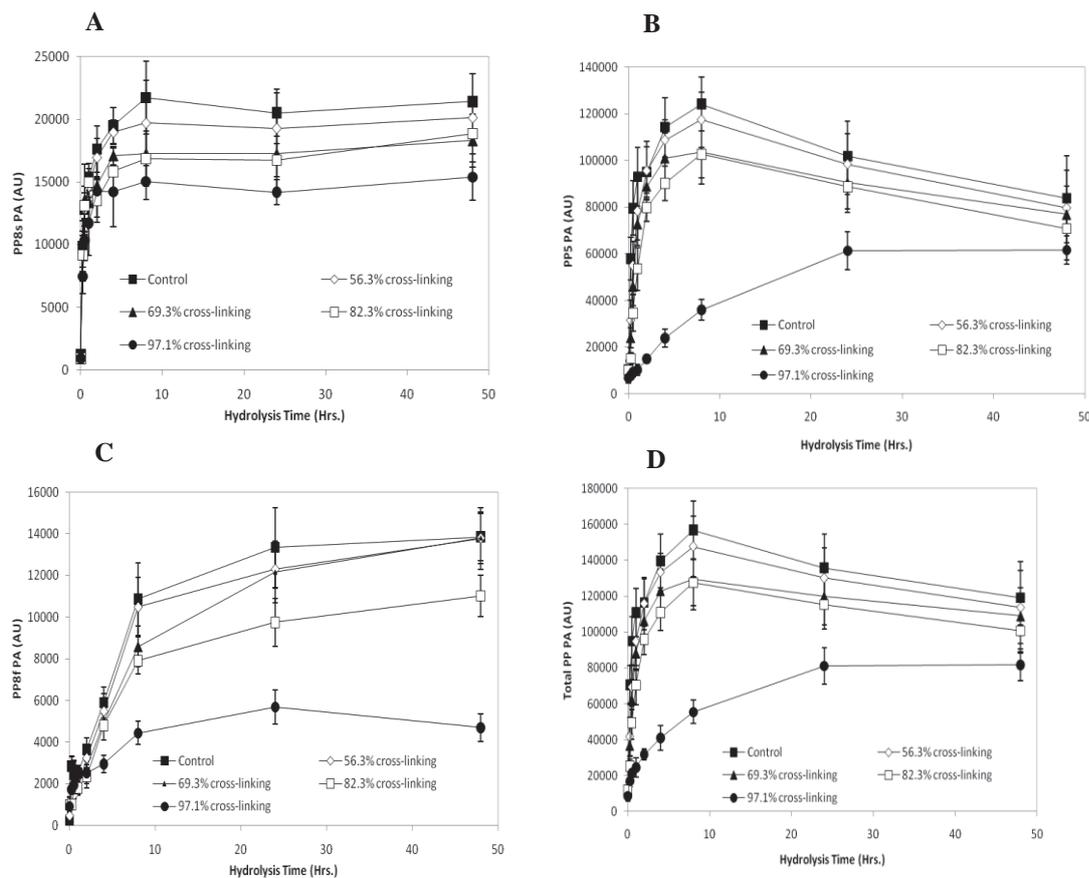


Figure 39. Complete hydrolysis patterns of  $\beta$ -casein as observed from RP-HPLC quantification: A, PP8slow; B, PP5; C, PP8fast; D, total proteose peptones. Areas under the peaks for the different peptides were calculated and presented as an increase in the peptide concentration. Error bars refer to standard deviations. Note scale differences.

The effect of TG-induced cross-linking became clear in the complete hydrolysis patterns. With an increase in cross-linking, there was a decrease in the release of all protease peptones. For PP8slow and PP8fast, the effect became clear in the secondary stage of hydrolysis, i.e. mainly after 2 h of hydrolysis in the complete hydrolysis patterns (Figure 39); in contrast, for PP5 and total proteose peptones, the effect was clear only in the primary stage of hydrolysis (Figure 37).

The results from both analyses were quite similar and showed decreases in plasmin-induced hydrolysis with rates of 1.937 ( $R^2 = 0.9825$ ) and 1.7566 ( $R^2 = 0.8712$ ) for SDS-PAGE and RP-HPLC respectively. The slightly higher rate of decrease for SDS-PAGE than for RP-HPLC could have been due mainly to minor underestimation by SDS-PAGE and/or minor overestimation by RP-HPLC.

There are two main reasons for the negative effect of TG-induced cross-linking.

1. Lysine availability: Plasmin hydrolyses after lysine or arginine residues, preferentially lysine; in the transglutamination reaction, lysine is the secondary target that is involved in the cross-linking reaction. According to Christensen, Sørensen, Højrup, Petersen, and Rasmussen (1996), TGs are much less selective towards amine donor lysine residues in proteins than they are towards the glutamine substrate. The cross-linking of lysine with a glutamine residue results in modification of the lysine residue, making it unrecognisable to the substrate specificity pocket of plasmin and, ultimately, the rate of plasmin-induced hydrolysis decreases.

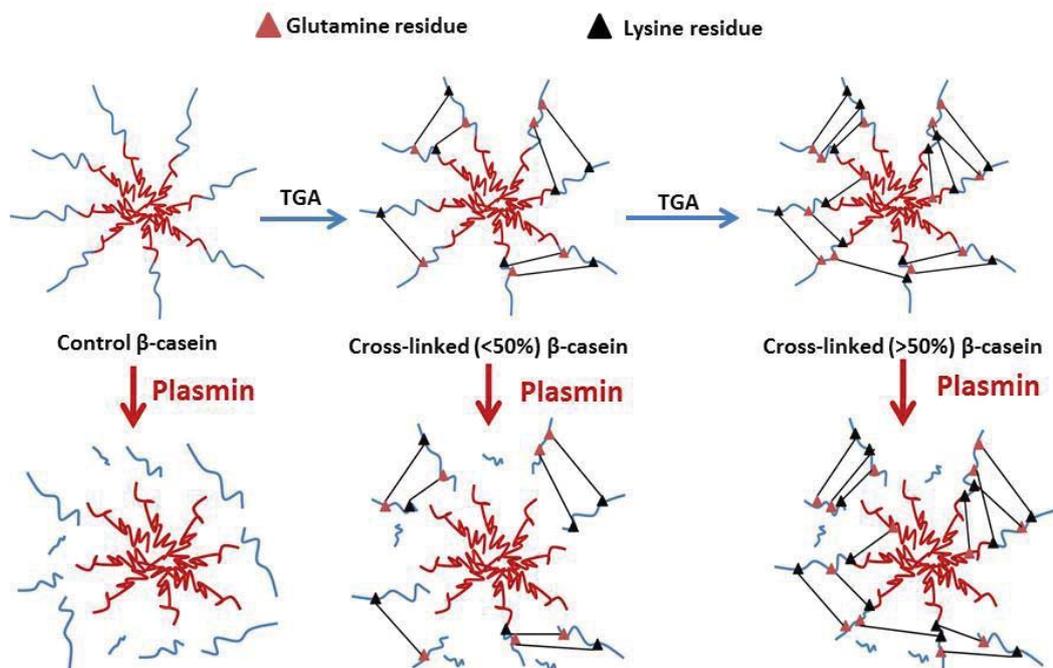


Figure 40. Resistance of TG-cross-linked  $\beta$ -casein to plasmin-induced hydrolysis as measured. Cross-linking made the protein unrecognisable to plasmin through lysine capping and the cross-linking affected the release of hydrolysed peptides.

2. Cross-linking effect: The cross-linking effect could also play a very important role in the decrease in the measured hydrolysis rate. In the study, the hydrolysis rate was measured by quantifying the peptides released; if these peptide fractions were already cross-linked, they would not be released after hydrolysis and,

ultimately, we would observe a decrease in the hydrolysis rate because of the release of fewer hydrolysed peptides.

There was not much effect on the plasmin-induced hydrolysis at lower extents of cross-linking, i.e. < 56% cross-linking, but the effect became almost linear with further increases in cross-linking. These effects could have been because lysine residues are the secondary target in transglutamination reactions and glutamine residues are the primary target. At low extents of cross-linking, as there might be less involvement of lysine residues, there might be less modification of lysine and therefore less effect on the hydrolysis; with an increase in the extent of cross-linking, the involvement of lysine residues might increase, with a resulting linear decrease in the hydrolysis.

For SDS-PAGE analysis, the hydrolysis rate was determined by the measurement/quantification of  $\gamma$ -casein. Because of the formation of cross-links by TG, cross-linked species of  $\gamma$ -casein could possibly be formed and could play a role in underestimating the generation of  $\gamma$ -casein. However, there appears to be little chance of the formation of cross-linked  $\gamma$ -casein species because, according to Christensen et al. (1996), Gln54, Gln56, Gln72, Gln79 and Gln182 are the sites of TG-induced cross-linking in  $\beta$ -casein and all except Gln182 are in the hydrophilic region of  $\beta$ -casein; although this indicates little chance of the involvement of  $\gamma$ -casein ( $\beta$ -casein f105/107–209) in cross-linking, it cannot be ruled out.

For RP-HPLC analysis, the hydrolysis rate was determined by the quantification of proteose peptones. Because of the formation of cross-linked species of proteose peptones, especially PP5 ( $\beta$ -casein fN–105/107) as observed in Figure 36, there was a broadening of the peptide peak or a widening of the baseline. Therefore, there was the possibility of a slight overestimation of the proteose peptone concentration; however, this was minimal and did not affect the quantification. The formation of cross-linked PP5 species was clearly observed in Figure 36, which could have been due to the presence of all TG-induced cross-linking sites except Gln182 in PP5 (Christensen et al., 1996).

### 5.4.3 A comparative analysis

The results from the present study were compared with the previous studies on the effects of succinylation (Chapter 3) and lactosylation (Chapter 4) on plasmin-induced hydrolysis, to achieve a better understanding of the factors that affect the hydrolysis. For the comparative study, only the primary (initial) hydrolysis rates, when  $\beta$ -casein was the primary substrate and was hydrolysed into  $\gamma$ -casein and proteose peptones, were compared.

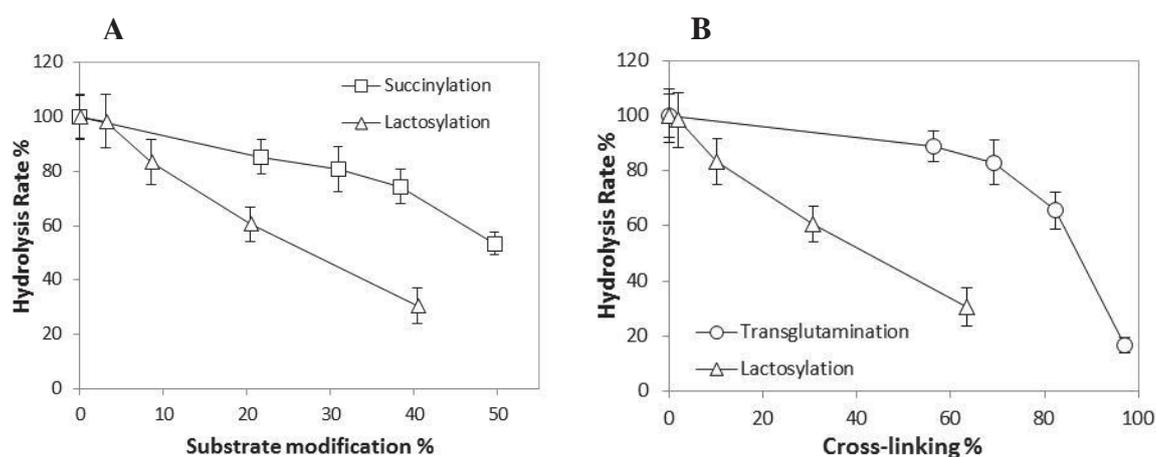


Figure 41. Comparative analysis of the effect of substrate modification on plasmin-induced hydrolysis. A. Comparative effect of lactosylation and succinylation with respect to loss of the  $\epsilon$ -amino group of the lysine residue. B. Comparative effect of lactosylation and transglutamination with respect to the extent of cross-linking of the protein chain. Error bars refer to standard deviations.

The extent of substrate modification was measured using two different factors: (1) loss of the free amino group, as measured by the fluorescamine method; (2) extent of cross-linking, as measured using SDS-PAGE. Lactosylation was compared with succinylation based on loss of the free amino group (Figure 41A), whereas lactosylation was compared with transglutamination based on the extent of cross-linking (Figure 41B).

From the previous studies, it was concluded that two factors were mainly involved in the negative effect of substrate modification on plasmin-induced hydrolysis.

1. Effect of lysine modification – succinylation, lactosylation.
2. Effect of cross-linking – transglutamination, Maillard-induced cross-linking.

When the effects of lactosylation and succinylation on plasmin-induced hydrolysis were compared in terms of loss of the free amino group, it was found that lactosylation had a much greater effect, having a steeper linear decrease (-1.773) than succinylation (-0.8705) (Figure 41A, Table 6).

Table 6. Effect of substrate modification on plasmin-induced hydrolysis rate of  $\beta$ -casein

Treatment	Effect on hydrolysis rate as measured by RP-HPLC
Lactosylation – lysine modification	-1.77
Succinylation – lysine modification	-0.87
Lactosylation – cross-linking	-1.10
Transglutamination – cross-linking	-0.69

With reference to the extent of cross-linking, the effect of lactosylation was much greater than the effect of transglutamination on the plasmin-induced hydrolysis rate. The decrease in the hydrolysis rate was much greater for lactosylation (-1.0975) than for transglutamination (-0.689). For lactosylation, the decrease was linear and even a small amount of modification had an effect, whereas, for transglutamination, the effect became visible only when the extent of cross-linking was around 50%. Interestingly, when the TG-induced cross-linking was increased from 50% to more than 95%, the decrease in the hydrolysis rate became linear. Both succinylation and transglutamination had a negative effect on plasmin-induced hydrolysis but the effect of lactosylation was much greater (Figure 41, Table 6).

Modification of lysine residues by substrate modification affected plasmin-induced hydrolysis negatively in a linear manner. However, when  $\beta$ -casein was cross-linked by TG to achieve Maillard-like cross-linking, these results suggested that, for a negative effect on plasmin-induced hydrolysis, at least 50% TG-induced cross-linking was required, which was markedly higher for all except the most highly lactosylated sample. It is also important to note that, even in TG-induced cross-linking, lysine residues are the target in the cross-linking. Therefore, on the basis of this comparative study (Figure 41, Table 6), the modification of lysine, by the formation of either lactulosyl-lysine or succinyl-lysine at lower degrees of lactosylation and/or cross-linking of the same residue at higher degrees of lactosylation or transglutamination, is the main reason for the decrease in the rate of plasmin-

induced hydrolysis. Although the Maillard-induced cross-linking was not sufficient at higher degrees of lactosylation to substantially affect the release of hydrolysed peptides when compared with transglutamination (Figure 41B, Table 6), the effect of cross-linking cannot be ruled out for lactosylation.

## 5.5 CONCLUSIONS

The results clearly indicated that transglutamination affected plasmin-induced hydrolysis negatively. The involvement of lysine in cross-linking made it unrecognisable to the substrate-binding pocket of plasmin and the cross-linking prevented the release of hydrolysed peptides. Thus, transglutamination may be useful for controlling the plasmin-induced hydrolysis of milk proteins, to minimise the texture- and flavour-related defects that are caused by the release of hydrolysed peptides. Comparison with other means of substrate modification indicated that lysine modification played a major role in minimising plasmin-induced hydrolysis compared with cross-linking. The results indicated that modification of lysine affects plasmin-induced hydrolysis negatively, to various degrees depending on the type of modification. Lactosylation was found to have a greater effect at the same level of lysine modification than succinylation and transglutamination in terms of cross-linking. A mechanism for this phenomenon is proposed (Chapter 4). Lactosylation involves the attachment of lactose and, in advanced stages, cross-linking, thus modifying lysine and making it unrecognisable to plasmin; in addition, the cross-linking may affect the release of plasmin-generated peptides. Transglutamination also modifies lysine by cross-linking; it has a similar effect to lactosylation, but to a lesser extent. In contrast, succinylation modifies the charge associated with lysine, making it unrecognisable to plasmin. Cross-linking mainly affected the release of the hydrolysed peptides and at least 50% TG-induced cross-linking was required to observe a negative effect on plasmin-induced hydrolysis. The present study explains the different effects of each type of substrate modification and this knowledge can be used to make protein resistant to plasmin-induced hydrolysis.



# Chapter 6: Effect of micellar structure of caseins and their modifications on plasmin-induced hydrolysis

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## 6.1 INTRODUCTION

Plasmin is an indigenous milk enzyme that causes the breakdown of milk proteins in a wide variety of dairy products; such hydrolysis of milk proteins is a major concern in the dairy industry as it leads to defects in the texture and taste, e.g. gelation and bitterness, of various dairy products. Different approaches to control plasmin-induced hydrolysis in milk products have been reported. Substrate modification techniques such as lactosylation (Bhatt et al., 2014; Dalsgaard et al., 2007), transglutamination (Chapter 5) and succinylation (Chapter 3) have been investigated in a pure protein model system to control plasmin-induced hydrolysis by decreasing the availability of the substrate. As plasmin hydrolyses proteins on the carboxyl site of Lys–X and Arg–X bonds, with a preference for the Lys–X bond, lysine residues on the  $\beta$ -casein backbone were targeted in previous studies on a  $\beta$ -casein model system (Bhatt et al., 2014); it was found that substrate modification targeting the  $\epsilon$ -amino group of lysine can be useful in blocking plasmin's action on  $\beta$ -casein and a mechanism has been proposed (Chapters 3, 4 and 5).

The structural and assembly aspects of caseins are different in a pure protein system from those in a real milk system. The caseins in bovine milk are present in the form of a colloidal dispersion known as the casein micelle (Schmidt, 1982). The casein micelle has a diameter of 50–500 nm (average  $\sim$  150 nm), has a molecular weight ranging from  $10^6$  to  $10^9$  Da (average  $\sim$   $10^8$  Da), comprises approximately  $10^4$  casein molecules and contains inorganic matter, mainly calcium phosphate, about 8 g/100 g of casein (Walstra et al., 2005). The casein micelle is composed of the four main caseins:  $\alpha_{s1}$ -casein ( $\alpha_{s1}$ -CN),  $\alpha_{s2}$ -CN,  $\beta$ -CN and  $\kappa$ -CN (molar ratio 4:1:4:1 respectively) (de Kruif & Holt, 2003; Swaisgood, 2003). Plasmin hydrolyses primarily  $\beta$ -CN and  $\alpha_{s2}$ -CN; the hydrolysis of  $\alpha_{s1}$ -CN is slower and  $\kappa$ -CN is more

resistant to hydrolysis (Andrews, 1983; Andrews & Alichanidis, 1983; Chen & Ledford, 1971). The presence of the caseins in this micellar structure could have different effects on their accessibility and hydrolysis by plasmin and modification of the caseins inside the micelle could affect its structure and the accessibility of different caseins to plasmin. The effect of the modification of caseins in the casein micelle and alteration of the micellar structure on plasmin-induced hydrolysis has not been studied.

The present study investigated the effect of protein modification in the micellar system, along with alteration of the casein micelle structure, on plasmin-induced hydrolysis in a skim milk system. Protein modification was achieved by attaching a succinate group at the  $\epsilon$ -amino group of lysine residues, leading to the formation of succinyl-lysine. The target sites were identified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The effects of different degrees of succinylation on the particle size in skim milk and on the dissociation of casein from the casein micelles were determined using a Zetasizer and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) respectively. The subsequent plasmin hydrolysis was monitored by quantifying the hydrolysed product using reverse-phase high performance liquid chromatography (RP-HPLC).

## **6.2 MATERIALS**

Skim milk was supplied by Fonterra Co-operative Group Ltd, Palmerston North. Plasmin from bovine plasma (5 U/mL) was purchased from Roche/Boehringer Mannheim GmbH (Germany). All chemicals for SDS-PAGE were purchased from Merck (Darmstadt, Germany). An acrylamide/bisacrylamide mixture 37.5:1 (2.6% C) from Bio-Rad (Hercules, CA, USA) was used. Water-free acetone was from Thermo Fisher Scientific (Auckland, New Zealand). Succinic anhydride and fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'dione) were purchased from Sigma–Aldrich (Auckland, New Zealand).

## **6.3 METHODS**

### **6.3.1 Succinylation of skim milk**

Skim milk was adjusted to pH 6.7. A small quantity (0.02% w/w) of sodium azide was added to the skim milk samples as a preservative. Different degrees of succinylation were obtained by adding different amounts of succinic anhydride to continuously stirred subsamples of skim milk at room temperature (25°C) and maintaining the pH at 6.7 by the addition of 1 N NaOH. The degree of succinylation was measured using the fluorescamine method, as described by O'Connell and de Kruif (2003).

### **6.3.2 Measurement of degree of succinylation**

The level of reactive amino groups ( $\epsilon$ -amino groups of lysine residues) in the protein was measured using the fluorescamine method (De Bernardo et al., 1974; O'Connell & de Kruif, 2003; Udenfriend et al., 1972), as described in Section 3.3.2.

### **6.3.3 RP-LC–MS/MS analysis**

Skim milk samples with different degrees of succinylation were digested with trypsin in 0.2 M ammonium bicarbonate buffer (pH 8.1), at 37°C for 4 h, using an enzyme: substrate ratio of 1: 25 (w/w), followed by inactivation of the trypsin by

the addition of 10  $\mu$ L of 50% formic acid. RP-LC–MS/MS analysis and protein identification were carried out as described in Section 3.3.3.

#### **6.3.4 Turbidity measurement**

The turbidity of different succinylated skim milk samples was determined by measuring the absorbance at 700 nm using a UV spectrophotometer (Japan Spectroscopic Co., Hachioji City, Japan) and a cell with a 2 mm light path. All turbidity measurements were carried out after 40 times dilution of the sample with calcium imidazole buffer (20 mM imidazole, 5 mM CaCl<sub>2</sub>, 30 mM NaCl, pH 7.0). The measurements were carried out in triplicate and average values were used to draw the plot.

#### **6.3.5 Particle size measurement**

Particle size measurements were performed to observe the effect of succinylation on the casein micelle size distribution in skim milk samples. The particle size was measured by photon correlation spectroscopy using a Malvern Zetasizer 4 instrument and the associated ZET5110 particle sizing cell (Malvern Instruments Ltd, Malvern, Worcs., UK) at 20°C, by the method of Anema and Li (2003a). Skim milk samples (50  $\mu$ L) were dispersed in 5 mL of calcium imidazole buffer before particle size measurement, as described by Anema (1997).

#### **6.3.6 Dissociation of milk proteins from casein micelles**

Centrifugation was used to identify the level of casein dissociation from the micelle into the serum phase and to identify the association of whey proteins with the casein micelles. The centrifugation was done using a method that has been described and used previously (Anema, 2007; Anema, Lee, & Klostermeyer, 2007; Anema, Lee, Lowe, & Klostermeyer, 2004; Rodriguez del Angel & Dalgleish, 2006). A subsample (1 mL) of each of the heat-treated milk samples was transferred into an Eppendorf tube (total volume 1.5 mL) and centrifuged at 14,000 rev/min (25,000 *g* average) for 1 h at 20°C in an Eppendorf centrifuge Type 5417C (Eppendorf AG, Hamburg, Germany). Non-sedimentable whey proteins were

defined as serum whey proteins (Anema, 2007). The depletion of caseins from the micelle was measured by carefully removing the clear supernatant phase and analysing by the SDS-PAGE method of Anema and Klostermeyer (1997); this was represented as a percentage of the total protein.

### **6.3.7 Plasmin-induced hydrolysis**

The skim milk samples were hydrolysed by plasmin added at 15  $\mu\text{L}/\text{mL}$  (0.1 U/mL) of skim milk. Skim milk samples were incubated in a water bath at 37°C and were allowed to equilibrate at temperature for 1 h before plasmin addition; the hydrolysis pattern was observed at different incubation times of 0 min, 15 min, 30 min, 1 h, 2 h and 4 h. The samples were removed after each incubation time, and the reaction was stopped immediately by the addition of an equal amount of 12% trichloroacetic acid (TCA).

### **6.3.8 AU-PAGE**

Skim milk samples with different levels of hydrolysis were diluted 1:40 with alkaline urea sample buffer and were analysed by alkaline urea polyacrylamide gel electrophoresis (AU-PAGE) (Creamer, 1991a). A Mini Protean II system (Bio-Rad Laboratories, Richmond, CA, USA) was used and the gels were run at 210 V and 70 mA for approximately 1.7 h followed by staining and de-staining; scanning of the gels was carried out in the same way as in the SDS-PAGE method (Section 3.3.5).

### **6.3.9 RP-HPLC**

The plasmin-induced hydrolysis of different succinylated milk samples was analysed by measuring the peptides that were soluble in 6% (w/v) TCA using RP-HPLC. The 6% TCA was used to precipitate the macromolecules, e.g. different caseins and whey proteins.

### ***Preparation of 6%-TCA-soluble extracts***

6%-TCA-soluble extracts were prepared by the addition of 12% TCA to the sample in a 1:1 ratio to give a final concentration of 6% TCA and allowing the mixtures to stand for 2 h at room temperature. The mixtures were then centrifuged at 14,000 rev/min for 15 min and the supernatants were collected to yield the 6%-TCA-soluble extracts.

### ***Analysis of peptides by RP-HPLC***

HPLC analysis was performed on Agilent 1100 series equipment with a UV/VIS photodiode array multi-wavelength detector using a 250 mm x 4.6 mm reversed-phase column (Phenomenex-Jupiter 300 C18, 5  $\mu$ m) at 40°C and a binary solvent gradient system at a flow rate of 1 mL/min and detection at 210 nm. Solvent A: 0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water and solvent B: 0.1% (v/v) TFA in HPLC-grade acetonitrile were used.

The proportion of solvent A to solvent B was constant at 98:2 for the first 5 min; then solvent B was increased from 2% to 15% during the next 15 min, then increased to 65% in the next 35 min, maintained for 5 min and finally decreased back to 2% solvent B in the next 5 min in readiness for the next sample injection. Injections of 50  $\mu$ L of filtrates were made by auto-injector. All samples were analysed in duplicate and data analysis was computed by Agilent (Hewlett Packard) Chemstation software. The peptides were quantified by integration of peak areas.

All experiments reported were repeated at least twice with the same milk samples. In addition, the experiments were repeated with several different milk samples. Although there were some variations between individual milks, the same trends and relationships as reported here were found for all samples examined to date.

## 6.4 RESULTS AND DISCUSSION

### 6.4.1 Characterisation of succinylated samples

#### *Degree of succinylation*

Skim milk samples were modified by adding different amounts of succinic anhydride to achieve different degrees of chemical modification. The degree of succinylation was measured in terms of a decrease in the reactive amino groups, i.e. the  $\epsilon$ -amino group of lysine, using the fluorescamine method (Section 3.3.2). The degree of succinylation results are summarised in Table 7.

Table 7. Degree of succinylation as measured by the fluorescamine method

Succinic anhydride (g/g of protein)	Skim milk succinylation (%)
0	0
0.01	10.35
0.05	20.13
0.075	36.13
0.1	43.60

Table 7 shows that the number of reactive  $-\text{NH}_2$  groups decreased with an increase in the concentration of succinic anhydride and this was used as an indicator of the degree of succinylation (%). The skim milk samples were characterised into five different degrees of succinylation.

In milk systems, caseins are unstructured (mostly random coiled), in comparison with the globular whey proteins. The lack of defined tertiary structure in caseins facilitates the accessibility of the  $\epsilon$ -amino group of lysine; additionally, the  $\epsilon$ -amino group has a relatively low  $\text{pK}_a$  value (Franzen & Kinsella, 1976), allowing it to readily undergo succinylation. In skim milk samples, caseins are present in a structured micellar form and their reactivity/exposure might be less than in systems in which they are present in non-micellar forms such as sodium caseinate or pure protein solutions (such as a  $\beta$ -CN model system) (Chapter 3).

### ***Effect of succinylation on electrophoretic migration pattern***

Succinylation was found to affect the electrophoretic migration pattern of proteins, as was observed for the  $\beta$ -CN model system (Chapter 3); with an increase in the degree of succinylation, the pattern of the  $\beta$ -CN band on SDS-PAGE was modified (Chapter 3). In the present study, all of the skim milk proteins were found to be modified by succinylation when analysed by AU-PAGE (Figure 42). The  $\beta$ -CN and  $\alpha_5$ -CN bands migrated faster with an increase in the degree of succinylation and the effect was greater for  $\beta$ -CN than for  $\alpha_5$ -CN. The faster migration of the proteins on AU-PAGE could have been due mainly to the change in the net charge of the protein. Succinylation increases the net negative charge by replacing the positive charge of the  $\epsilon$ -amino group of lysine with a negatively charged succinate group (carboxylate group) (Franzen & Kinsella, 1976). The current observations are in agreement with Habeeb et al. (1958) and Schwenke et al. (1981), who observed an increase in the electrophoretic mobility (anodic migration) in alkaline media with an increase in succinylation. The generation of multiple species from each protein, caused by succinylation, was clearly seen on AU-PAGE (Figure 42); as the degree of succinylation increased, there was a progressive increase in both the number of bands associated with a particular protein fraction and the width of each band. This indicated that, for each sample, although a single degree of succinylation was reported, this was an average and there was in fact a range of degrees of succinylation for each protein. In contrast, the control had only a single discrete band.

In the present study, the sharpness of the different protein bands on AU-PAGE was found to decrease; this was in agreement with previous studies (Chapter 3; Ma et al., 2009), in which the sharpness of the protein bands decreased on SDS-PAGE. This phenomenon could have been due to the generation, through succinylation, of more heterogeneous populations of protein, as described earlier; alternatively, the decreased staining intensity of succinylated protein could also have been due to an increase in the net negative charge by the added carboxylate

groups, which are dye repulsive and thus bind less dye (Hoagland, 1966), as observed for succinylated  $\beta$ -CN (Chapter 3).

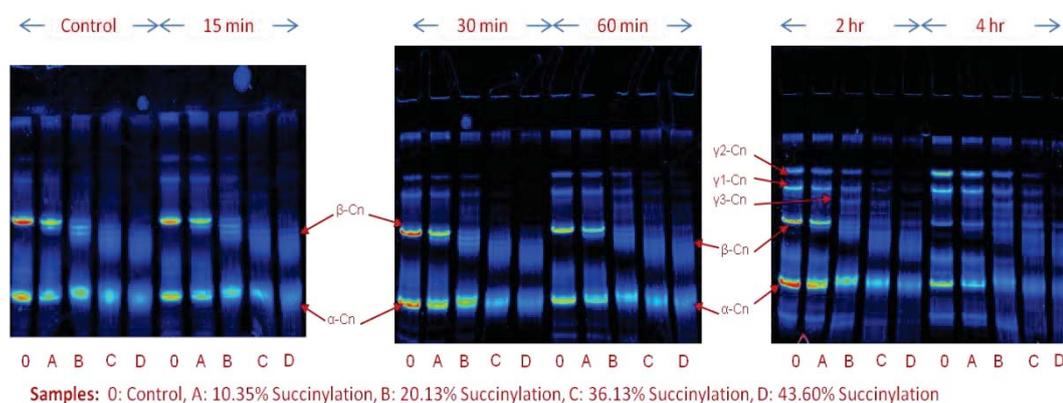


Figure 42. Effect of succinylation of skim milk on plasmin-induced hydrolysis patterns of different proteins on AU-PAGE. Control: no hydrolysis; 15 min, 30 min, 60 min, 2 h and 4 h represent the hydrolysis times.

It was difficult to analyse the hydrolysis pattern using AU-PAGE because of the modification of the protein by succinylation; however, it was observed that the peptide bands generated –  $\gamma_{(1,2,3)}$ -CNs – decreased, compared with the control sample, with an increase in the degree of succinylation (Figure 42). This decrease can be attributed to the decrease in hydrolysis and the decrease in the staining intensity and/or the presence of multiple degrees of succinylation for each protein species. However, it is important to note that  $\gamma$ -CN has only one succinylation site, Lys113, as observed by RP-LC–MS/MS analysis (Table 9); therefore, the chances of the formation of multiple species and decreases in the staining intensity, due to succinylation, are less in  $\gamma$ -CN but cannot be ruled out. For a thorough analysis of the hydrolysis pattern, the RP-HPLC technique was used to overcome the problems faced with AU-PAGE.

### ***Identification of succinylation sites for different proteins***

The sites of succinylation on the different proteins in skim milk were identified using RP-LC–MS/MS analysis. Milk samples with either a low (10%) or a high (43%)

degree of succinylation were analysed to identify the succinylation sites. The results are presented in Table 8 and Table 9.

Electron spray ionisation (ESI)-MS/MS analysis of the tryptic digests of succinylated milk samples following HPLC purification enables the different protein sequences and the modified residues (Table 8 and Table 9) in which succinylation is occurring to be identified. The main objective of the present work was to identify target succinylation sites in the major milk proteins that are hydrolysed by plasmin, i.e.  $\alpha_{s1}$ -CN,  $\alpha_{s2}$ -CN,  $\beta$ -CN and  $\beta$ -lactoglobulin.

The succinylated species were identified by their mass change ( $\Delta m$ ), on succinylation, of 100.02 Da and the identified phosphorylation sites were found to be in agreement with those reported in the literature (Ginger & Grigor, 1999). A comparison of the MS/MS spectra between the milks with different degrees of succinylation allowed us to identify signature peptides associated with succinylation. Typical examples of MS/MS spectra of the tryptic digests of the low and high succinylated skim milk samples are shown in Figure 43. In Figure 43A, peak 1839.87 was present in the 43.60% succinylated sample, representing succinylated  $\beta$ -CN(100–113) with two succinylation sites, Lys105 and Lys107, but was absent in the 10.35% succinylated sample. In contrast, in Figure 43B, peak 1605.80 was present in both succinylated samples, representing  $\alpha_{s1}$ -CN(100–113) with one succinylation site at Lys124.

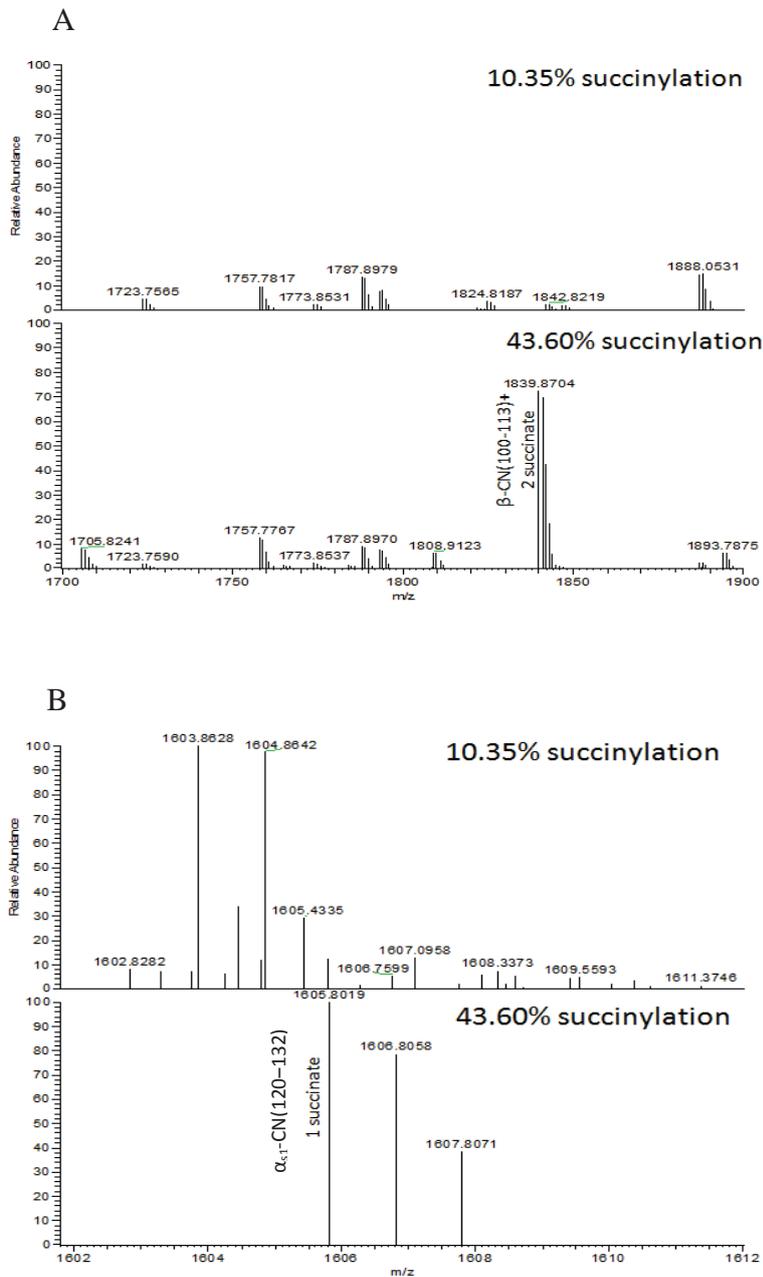


Figure 43. Typical MS/MS spectra of the tryptic digests of 10.35% and 43.60% succinylated skim milk samples. A. Peak 1839.87, present in the 43.60% succinylated sample, represents succinylated  $\beta$ -CN(100–113) with two succinylation sites, Lys105 and Lys107, which is absent in the 10.35% succinylated sample. B. Peak 1605.80, present in both succinylated samples, represents  $\alpha_{s1}$ -CN(120–132) with one succinylation site at Lys124.

As expected, fewer residues were succinylated at lower degrees of succinylation and the numbers increased as the degree of succinylation increased. In the skim milk samples with 10.35% succinylation, the succinylation occurred specifically at Lys7, Lys34, Lys83 and Lys124 in  $\alpha_{s1}$ -CN, Lys80 and Lys165 in  $\alpha_{s2}$ -CN, Lys99 and Lys107 in  $\beta$ -CN and Lys100 in  $\beta$ -lactoglobulin. In the skim milk samples with 43.60% succinylation, the succinylation occurred at Lys7, Lys34, Lys36, Lys42, Lys83 and Lys124 in  $\alpha_{s1}$ -CN, Lys80, Lys150, Lys152, Lys158 and Lys165 in  $\alpha_{s2}$ -CN, Lys28, Lys29, Lys32, Lys99, Lys105, Lys107 and Lys113 in  $\beta$ -CN and Lys75, Lys77, Lys83, Lys91, Lys100, Lys135 and Lys138 in  $\beta$ -lactoglobulin.

For all casein proteins, the number of succinylation sites increased as the degree of succinylation increased from 10.35 to 43.60%, with the number of sites for  $\alpha_{s1}$ -CN,  $\alpha_{s2}$ -CN and  $\beta$ -CN increasing from 4 to 6, 2 to 5 and 2 to 7 respectively, because the casein structure made these residues easily accessible.

At the higher degree of succinylation for  $\alpha_{s1}$ -CN, the absence of the succinylated peptides (1–22), (8–22), (43–58)P2, (59–79)P5 and (91–102) containing Lys3, Lys7, Lys58, Lys79 and Lys102 residues from the eluted material, coupled with the consideration that peptides containing C-terminal lysine can be generated only if the lysine residue is unmodified (Siciliano et al., 2000), ruled out these amino acids as potential sites of succinylation. Here, P2 and P5 represent the number of phosphorylations. Similarly, for  $\beta$ -CN, the absence of the succinylated peptides (29–48), (123–176) and (170–176) containing Lys32, Lys169 and Lys176 from the eluted material ruled out these lysine residues as potential succinylation sites.

Table 8. RP-LC-MS/MS analysis of 10% succinylated skim milk for site identification of succinylation following digestion with trypsin

Protein succinylation site	Sequence	Additional modification site	Charge	Experimental mass	Theoretical mass
$\alpha_{s1}$ B, D-CN	$\alpha_{s1}$ -CN(4-22): K.HPIK*HQGLPQEVLNENLLR.F		3	2334.26	2334.24
	$\alpha_{s1}$ -CN(23-36): R.FFVAPFPEVFGK*EK.V		3	1740.89	1740.88
	$\alpha_{s1}$ -CN (80-90): K.HIQK*EDVPSEY		2	1436.70	1436.69
	$\alpha_{s1}$ -CN (120-132): R.LHSMIK*EGIHAAQKQ.E		3	1605.80	1605.79
$\alpha_{s2}$ -CN	$\alpha_{s2}$ -CN(77-91): K.HYQK*ALNEINQFYQK.F		2	2022.99	2022.98
	$\alpha_{s2}$ -CN(161-166): R.LNFLK*K.I		2	861.50	861.50
$\beta$ -CN	$\beta$ -CN (98-105): K.VK*EAMAPK.H		2	972.50	972.49
	$\beta$ -CN (106-113): K.HK*EMPPPK.Y		3	1112.54	1112.53
$\beta$ -Lactoglobulin					
K100	$\beta$ -Lg (92-101): K.VLVLDTDYK*K.Y		2	1292.70	1292.69

Table 9. RP-LC-MS/MS analysis of 43% succinylated skim milk for site identification of succinylation following digestion with trypsin

Protein succinylation site	Sequence	Additional modification site	Charge	Experimental mass	Theoretical mass
$\alpha_{s1}$ B, D-CN	$\alpha_{s1}$ -CN(4-22): K.HPIK*HQGLPQEVLNENLLR.F		3	2334.26	2334.24
	$\alpha_{s1}$ -CN(23-34): R.FFVAPFPEVFGK*EK.V		3	1740.89	1740.88
	$\alpha_{s1}$ -CN(23-42): R.FFVAPFPEVFGK*EK*VNELSK.D	Phospho S41	2	2591.23	2591.22
	$\alpha_{s1}$ -CN(35-42): K.EK*VNELSK.D		2	1045.54	1045.53
	$\alpha_{s1}$ -CN(37-58): K.VNELSK*DIGESTEDQAMEDIK.Q	2 Phospho S41, 46	3	2697.08	2697.07
	$\alpha_{s1}$ -CN (80-90): K.HIQK*EDVPSEY		2	1436.70	1436.69
K124	$\alpha_{s1}$ -CN (120-132): R.LHSMIK*EGIHAAQKQ.E		3	1605.80	1605.79
$\alpha_{s2}$ -CN					
K80	$\alpha_{s2}$ -CN(77-91): K.HYQK*ALNEINQFYQK.F		2	2022.99	2022.98

K150, K152	$\alpha_{s2}$ -CN(150–158): K.K*TK*LTEEEK.N	2	1304.64	1304.63
K150, K152, K158	$\alpha_{s2}$ -CN(150–160): K.K*TK*LTEEEK*NR.L	2	1674.81	1674.79
K152	$\alpha_{s2}$ -CN(151–158): K.TK*LTEEEK.N	2	1076.53	1076.52
K165	$\alpha_{s2}$ -CN(161–166): R.LNFLK*K.I	2	861.50	861.50
<b><math>\beta</math>-CN</b>				
K28, K29	$\beta$ -CN (26–32): R.IINK*K*IEK.F	2	1071.59	1071.58
K32	$\beta$ -CN (29–48): K.KIEK*FQSEEQQTDELDQK.I	3	2659.17	2659.15
K29, K32	$\beta$ -CN (29–48): K.K*IEK*FQSEEQQTDELDQK.I	3	2759.19	2759.17
K32	$\beta$ -CN (30–48): K.IEK*FQSEEQQTDELDQK.I	2	2531.08	2531.06
K99	$\beta$ -CN (98–105): K.VK*EAMAPK.H	2	972.50	972.49
K99, K105	$\beta$ -CN (98–107): K.VK*EAMAPK*HK.E	2	972.50	972.49
K105	$\beta$ -CN (100–107): K.EAMAPK*HK.E	2	1010.49	1010.49
K105, K107	$\beta$ -CN (100–113): K.EAMAPK*HK*EMPPPK.Y	2	1839.86	1839.85
K107	$\beta$ -CN (106–113): K.HK*EMPPPK.Y	3	1112.54	1112.53
K107, K113	$\beta$ -CN (106–122): K.HK*EMPPPK*YPVEPFTER.Q	3	2331.11	2331.09
K113	$\beta$ -CN (108–122): K.EMPPPK*YPVEPFTER.Q	3	1965.93	1965.92
<b><math>\beta</math>-Lactoglobulin</b>				
K75	$\beta$ -Lg (71–77): K.IIAEK*TK.I	2	901.52	901.51
K75, K77	$\beta$ -Lg (71–83): K.IIAEK*TK*IPAVFK.I	2	1656.94	1656.93
K77, K83	$\beta$ -Lg (76–91): K.TK*IPAVFK*IDALNENK.V	2	2000.06	2000.06
K91	$\beta$ -Lg (84–100): K.IDALNENK*VLVLDTDYK.K	2	2062.06	2062.05
K100	$\beta$ -Lg (92–101): K.VLVLDTDYK*K.Y	2	1292.70	1292.69
K135	$\beta$ -Lg (125–141): R.TPEVDDEALEK*FDKALK.A	2	2047.02	2047.00
K135, K138	$\beta$ -Lg (125–141): R.TPEVDDEALEK*FDK*ALK.A	2	2147.03	2147.02
K138	$\beta$ -Lg (136–141): K.FDK*ALK.A	2	820.44	820.43

In this table, only the peptides giving fruitful information regarding sequence coverage and/or modified site localisation are included.

**K\*** modified lysine.

It was interesting to note that  $\beta$ -lactoglobulin had only one succinylation site (Lys100) at low degrees of succinylation but had seven sites at higher degrees of succinylation. This observation was in stark contrast to the succinylation patterns observed in the structured caseins. Such a marked increase in the succinylation sites of  $\beta$ -lactoglobulin could have been due to an increase in the unfolding and expansion of the protein with an increase in the degree of succinylation.  $\beta$ -Lactoglobulin is present as a globular protein in the milk system and most of the reactive residues are buried inside the structure; therefore, in its native state, prior to any degree of succinylation, only lysine residues on the outer surface of the protein would be available for succinylation. It would seem that, based on the data at low degrees of succinylation, only Lys100 lies on or in close proximity to the outer surface and thus is available for succinylation. However, as the degree of succinylation increases, because of the addition of the succinate (negatively charged) group at the lysine residue, there is a change in the balance of forces stabilising the tertiary structure and  $\beta$ -lactoglobulin unfolds, which results in the exposure of previously buried reactive residues and thus the number of succinylation sites ultimately increases.

Succinylation sites have not been identified previously for milk proteins. A more widely studied reaction that commonly occurs in milk systems is lactosylation, which involves the covalent attachment of lactose to the  $\epsilon$ -amino group of lysine residues to form lactulosyl-lysine. Lactosylation sites of milk proteins have been identified and are reported to be at Lys7, Lys34, Lys83, Lys103, Lys105, Lys132 and Lys193 for  $\alpha_{s1}$ -CN, at Lys32, Lys48, Lys107, Lys113 and Lys176 for  $\beta$ -CN (Scaloni et al., 2002) and at Lys47, Lys100, Lys14, Lys60, Lys135 and Lys138 for  $\beta$ -lactoglobulin (Arena et al., 2010; Fogliano et al., 1998).

The succinylation sites of the different milk proteins are quite different from the lactosylation sites. The difference in the modification sites for the two reactions could be due to the specificity of the reactions and/or to the changes in the structure and reactivity of different residues with the heat treatment for

lactosylation, compared with succinylation, which was carried out at 25°C, i.e. well below the denaturation temperature of  $\beta$ -lactoglobulin. However, in both reactions, the major lysine residues involved were present in the hydrophilic regions, presumably because of the accessibility of these residues for reaction.

#### **6.4.2 Changes in the micellar structure due to succinylation**

The effects of succinylation on the turbidity and the particle size of skim milk were measured and are presented in Figure 44. With an increase in the degree of succinylation, the turbidity of the skim milk sample decreased linearly ( $R^2 = 0.9838$ ) (Figure 44A) whereas the particle size increased (Figure 44B). The increase in particle size was observed with an increase in the degree of succinylation up to 36.13%. At 43.6% succinylation, the particle size decreased slightly. The increase in micellar size was greater at lower than at higher degrees of succinylation (Figure 44B). The mean count rate decreased markedly with an increase in the degree of succinylation.

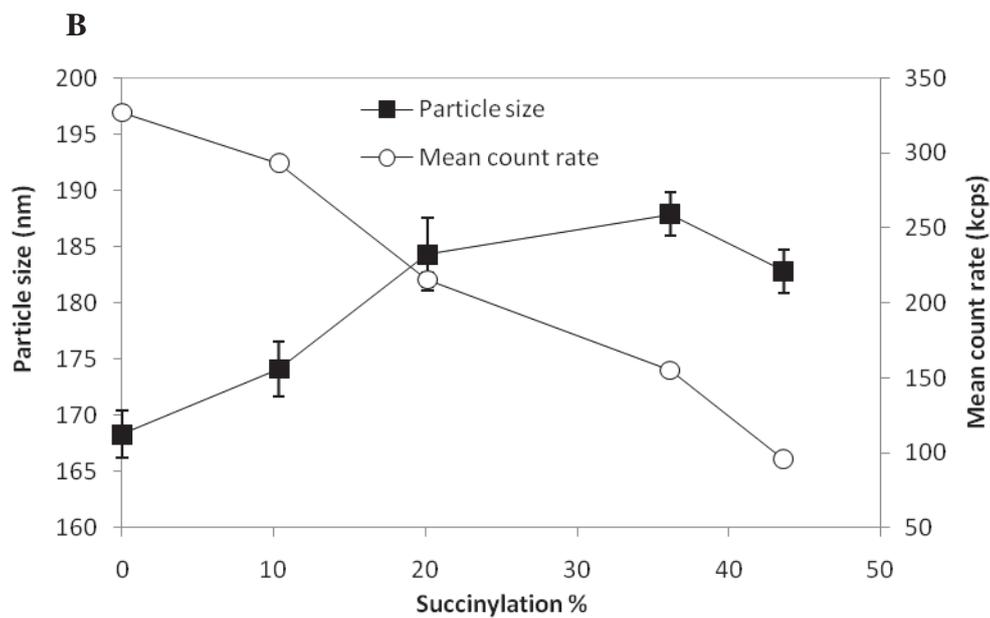
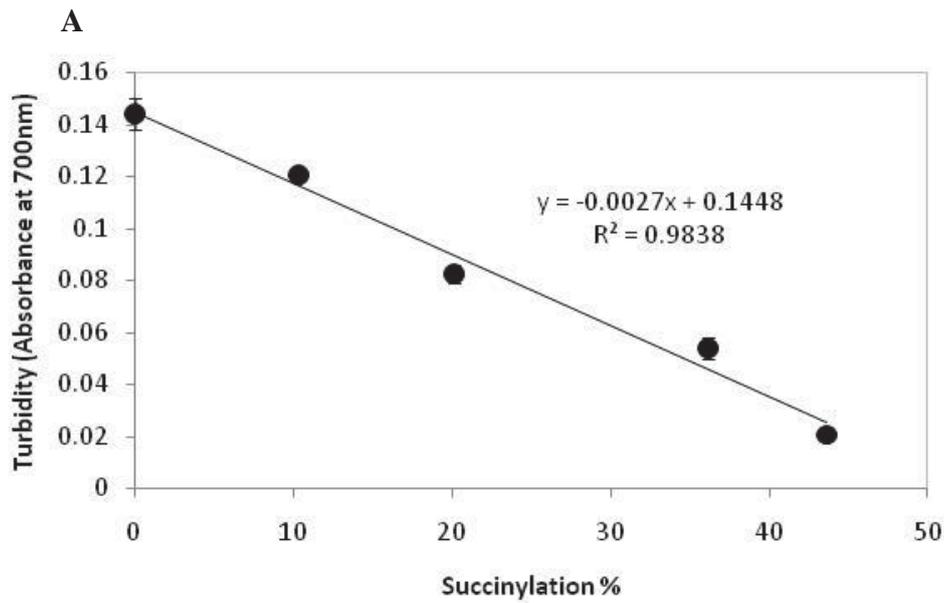


Figure 44. Effect of succinylation on: A, turbidity (as measured by absorbance at 700 nm); B, average particle size in skim milk. Error bars refer to standard deviations.

As the turbidity of a system depends on the size and the scattering factor of particles, we should observe an increase in turbidity with an increase in the particle size (Figure 44B). We observed the opposite, suggesting that there must be a change in the scattering factor of the particles. A change in the scattering factor

could be due to an increase in the voluminosity or the pore size, which results in a decrease in the density of the casein micelle.

At lower degrees of succinylation, the decrease in the turbidity can be attributed to the increase in the voluminosity and size of the micelle (Figure 44B), resulting from an increase in the negative charges on the protein molecules and thus electrostatic repulsions within the casein micelle, which in turn lead to a loosening of the structure. A decrease in the turbidity of milk with an increase in pH was observed by Anema and Klostermeyer (1997); they also attributed it to an earlier observation of an increase in the voluminosity of the micellar complex with increasing pH (Anema & Creamer, 1993; Creamer, 1985).

Succinylation results in the addition of a negatively charged succinate group at the  $\epsilon$ -amino group of the lysine residues on the protein chains. The addition of a negative charge results in a decrease in the surface hydrophobicity of the casein micelle (Lieske, 1999) and an introduction of short range repulsive forces because of the juxtaposition of two negatively charged carboxyl groups in the succinylated protein (Habeeb et al., 1958). Such decreases in the hydrophobicity, coupled with a concomitant increase in short range repulsive forces, could have been the reason for the observed swelling of the micelle or the dissociation of the casein micelle upon succinylation. A similar effect of increases in casein micelle size was observed in a previous study (Liu & Guo, 2008), in which the pH was increased from 6.0 to 12.0; the negative charge of the casein molecules increased, causing stronger electrostatic repulsion, which in turn led to a loosened casein micelle structure and thus an increase in the casein micelle size.

### ***Dissociation of proteins from the micelle***

With an increase in the degree of succinylation, dissociation of the caseins from the micelle was observed (Figure 45 and Figure 46); such disruption to the casein micelle system could result in a decrease in the turbidity. These results are in agreement with the decrease in the turbidity of the micelle observed during calcium chelation of milk (Appendix 1) and previously by other researchers studying

calcium-depleted micelle systems, in which the colloidal calcium phosphate was disrupted and concomitantly the casein micelles dispersed (Mizuno & Lucey, 2005; Odagiri & Nickerson, 1964; Pitkowski, Nicolai, & Durand, 2008).

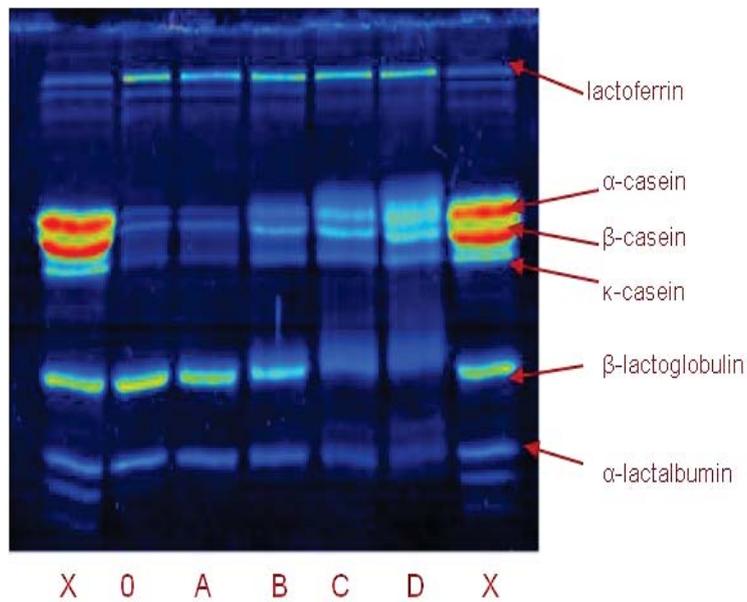


Figure 45. Casein micelle dissociation with increases in the degree of succinylation as measured using SDS-PAGE. Skim milk samples with different degrees of succinylation were centrifuged and the supernatants were loaded on SDS-PAGE. Samples: 0, control supernatant; A, 10.35% succinylation supernatant; B, 20.13% succinylation supernatant; C, 36.13% succinylation supernatant; D, 43.60% succinylation supernatant; X, control skim milk.

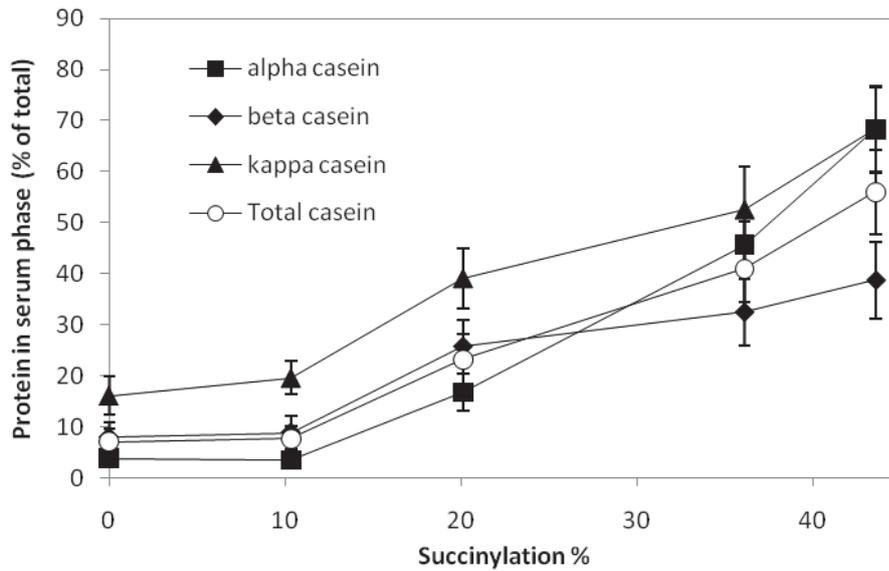


Figure 46. Effect of succinylation on dissociation of different caseins from the casein micelle. The dissociation was measured from the casein concentration in the serum phase. Error bars refer to standard deviations.

At lower degrees of succinylation, the increase in micelle size was probably due to increases in electrostatic repulsion (swelling of the micelle), whereas, at maximum succinylation, the slight decrease in micelle size could have been due to calcium depletion and micelle dissociation. It is clear from our results that the dissociation rate of  $\alpha$ -CN ( $\alpha_{s1}$ - +  $\alpha_{s2}$ -CN) was faster than that of  $\beta$ -CN; however, all caseins followed a fairly similar trend (Figure 46). The results are in accordance with previous studies (Lieske, Konrad, & Faber, 2000; Vidal et al., 1998), in which it was found that the amount of individual caseins in the serum increased with the degree of succinylation of the milk.

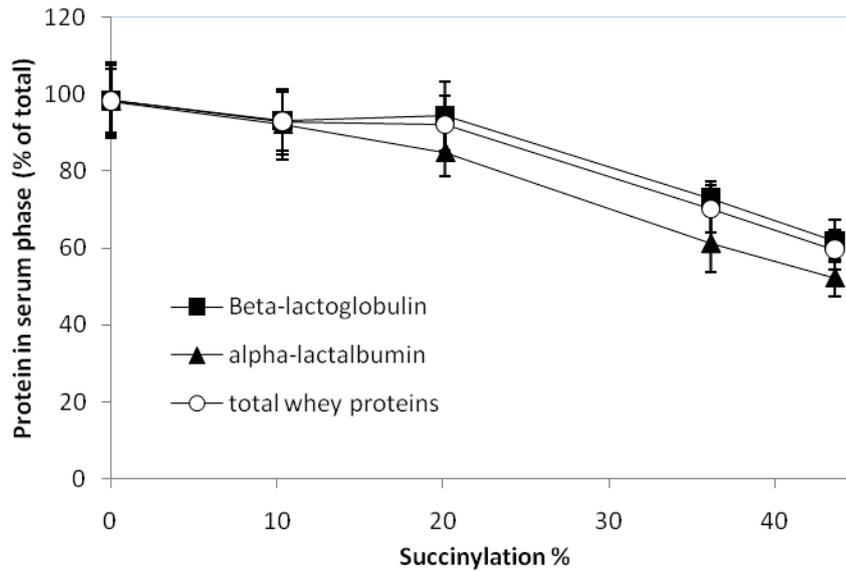


Figure 47. Effect of succinylation on the whey protein content in the serum phase. Error bars refer to standard deviations.

The effect of succinylation on soluble whey protein was also measured; the content of whey protein in the serum phase was found to decrease with an increase in succinylation (Figure 47). Both  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin followed a similar decreasing trend as a function of succinylation. It was noted that  $\alpha$ -lactalbumin decreased at a slightly faster rate than  $\beta$ -lactoglobulin. Upon succinylation, whey proteins unfold, lose their globular structure, aggregate by disulphide cross-linking with other whey proteins or  $\kappa$ -CN (Lieske & Konrad, 2001) and co-sediment in the pellet upon centrifugation, which could be the reason for the observed decreases in the whey protein content in the serum phase of the succinylated skim milk systems in this study.

#### 6.4.3 Effect of succinylation on plasmin-induced hydrolysis

The plasmin-induced hydrolysis of different succinylated skim milk samples was measured using an RP-HPLC technique. 6% (w/v)-TCA-soluble fractions were prepared and the increase in the peptide concentration with an increase in hydrolysis was measured for the different succinylated samples and compared with the control (non-succinylated sample).

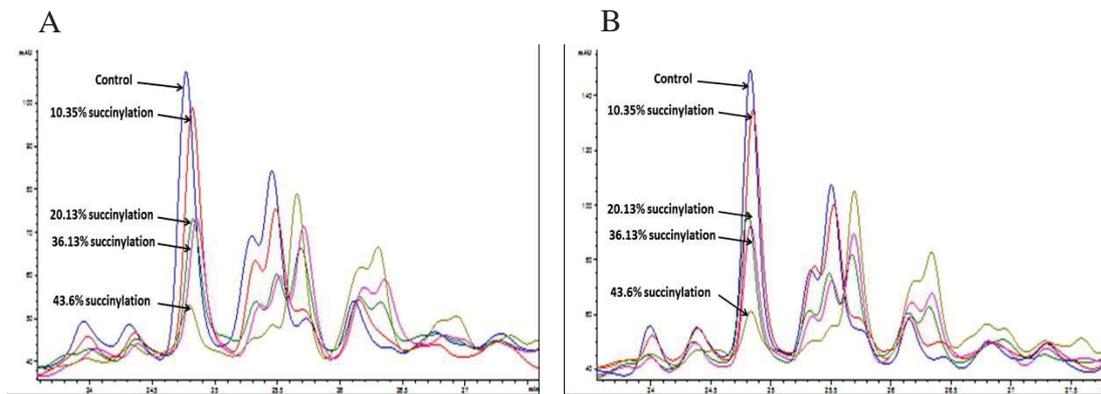


Figure 48. Comparative plasmin-induced hydrolysis patterns of skim milk succinylated to different degrees: A, after 30 min; B, after 2 h (as measured in 6% TCA fractions using RP-HPLC).

Figure 48 compares the plasmin-induced hydrolysis of different succinylated and non-succinylated skim milk samples. The peptide peak heights and areas decreased with an increase in the degree of succinylation (Figure 48), indicating a negative effect of succinylation on plasmin-induced hydrolysis. The elution patterns of the succinylated peptides differed from that of the control sample. These changes could have been due to changes in the charge and the hydrophobicity of the proteins and hydrolysed peptides, as observed in a previous study (Chapter 3).

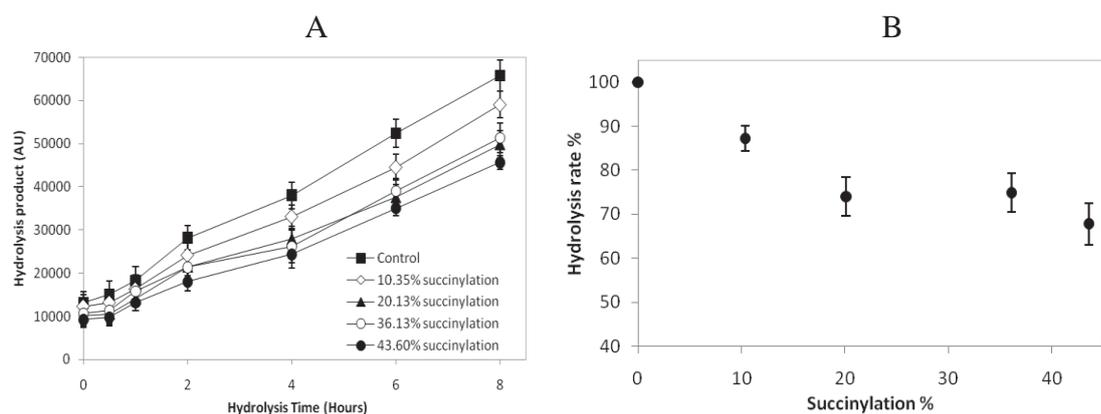


Figure 49. Effect of succinylation on plasmin-induced hydrolysis: A, effect on the release of hydrolysed peptides; B, effect on the rate of hydrolysis (as measured in 6% TCA fractions using RP-HPLC). Error bars refer to standard deviations.

With an increase in the hydrolysis time, the hydrolysed peptide peaks increased linearly (Figure 49A) and the rate of hydrolysis was calculated from the slope of the linear trend line. The plasmin-induced hydrolysis pattern of different caseins was found to decrease with increases in succinylation, as observed in a previous study (Chapter 3), which focused on a pure  $\beta$ -CN model system, but the trend of the rate change was different.

The rate of hydrolysis was found to decrease linearly with an increase in succinylation up to 20%. This decrease was probably due to the modification of the  $\epsilon$ -amino group of lysine residues through succinylation (Table 8 and Table 9), leading to the formation of succinyl-lysine and making lysine unrecognisable to the substrate-binding pocket of plasmin. A detailed mechanism for this has been proposed in a previous study (Chapter 3).

With further increases in succinylation, the decrease in the hydrolysis rate became stagnant up to 36% and then decreased slightly with further increases in succinylation up to 43.60% (Figure 49). This suggests that the major decrease in the hydrolysis rate was at lower succinylation and that the decrease was much less at higher succinylation. This observation is different from that in the previous study on a pure  $\beta$ -CN model system (Chapter 3), and can be attributed to the presence of other casein species and their association with  $\beta$ -CN in the casein micelle.

In the case of the  $\beta$ -CN model system,  $\beta$ -CNs self-associated in a simple micellar form at room temperature. The hydrophobic portion (C terminus of  $\beta$ -CN) formed the core of the pure  $\beta$ -CN micelle and the hydrophilic portion (N terminus) protruded from the micelle (Andrews et al., 1979; Aschi et al., 2009; de Kruif & Grinberg, 2002; O'Connell et al., 2003). The hydrophobic portion forms  $\gamma$ -CN and the hydrophilic portion forms proteose peptones upon hydrolysis by plasmin. In such a simple micellar system, the plasmin target sites, i.e. Lys105, Lys107 and Lys28/29, are easily accessible to plasmin and are readily hydrolysed. In contrast, in the more complex milk system,  $\beta$ -CN is present in a complex form with multiple

other caseins, e.g.  $\alpha_{s1}$ -CN and  $\alpha_{s2}$ -CN, known as the 'casein micelle'. The core of the casein micelle consists of equimolecular amounts of  $\beta$ -CN and  $\alpha_s$ -CN together with only small amounts of  $\kappa$ -CN, and the surface is composed of  $\kappa$ -CN and  $\alpha_s$ -CN and small amounts of  $\beta$ -CN (Dalgleish, Horne, & Law, 1989; Horne, 2006). The micelle is stabilised by the polyelectrolyte brush of  $\kappa$ -CN, which extends from the micellar surface to create a layer, estimated to be 5–10 nm thick, around the particles (de Kruif & Zhulina, 1996).  $\alpha_{s1}$ -CN,  $\alpha_{s2}$ -CN and  $\beta$ -CN are the primary targets of plasmin's action whereas  $\kappa$ -CN is quite resistant, making it difficult for plasmin to access the plasmin-sensitive proteins ( $\alpha_{s1}$ -CN,  $\alpha_{s2}$ -CN and  $\beta$ -CN); thus, these proteins are hydrolysed slowly compared with in an unstructured form, i.e. in pure protein systems.

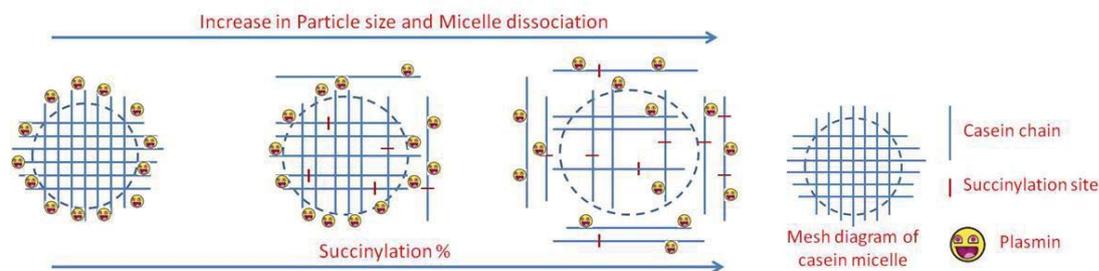


Figure 50. A simplistic mesh diagram of the casein micelle, indicating the effect of succinylation on plasmin-induced hydrolysis. Upon succinylation, particle size and micelle dissociation increase, affecting the pattern of plasmin-induced hydrolysis.

Upon succinylation, the attachment of succinate at Lys28, Lys29, Lys105, Lys107 and Lys113 (Table 8 and Table 9), as observed by RP-LC-MS/MS analysis, makes these residues unrecognisable to plasmin and there is a decrease in the hydrolysis rate. However, at the same time, the casein micelle swells and the voluminosity and/or porosity (mesh size) of the micelle increases (Figure 50), as observed by the increase in particle size and the decrease in turbidity (Figure 44), along with the dissociation of the caseins from the micelle. Calculations on estimated mesh size of the casein micelle and changes upon succinylation are shown in Appendix 7. Such changes in the casein micelle can facilitate access to the plasmin-sensitive proteins and could increase the hydrolysis rate. Further increases in the degree of succinylation lead to dissociation of the different caseins from the micelle, as observed using SDS-PAGE

(Figure 45 and Figure 46). This dissociation will increase the content of soluble casein and these dissociated caseins are readily hydrolysed by plasmin (Crudden et al., 2005b). Similar results of increase in the plasmin-induced hydrolysis of caseins were observed upon calcium chelation of the milk (see Appendix 1). Another reason for the increase in plasmin-induced hydrolysis could be the generation of an extended form, electrostatic repulsion and less aggregation of the proteins upon succinylation.

Association of whey protein with  $\kappa$ -CN was found to have a negative effect on plasmin-induced hydrolysis in milk, as observed in previous studies (Enright & Kelly, 1999). However, in the present study, succinylation opened up the casein micelle and caused the dissociation of  $\kappa$ -CN from the micelle; therefore, the effect of a whey protein coating around the micelle will not occur in succinylated milks and possibly may not play much role in affecting the action of plasmin on casein.

## 6.5 CONCLUSIONS

Succinylation increases the negative charges on casein, resulting in a decrease in the turbidity and mean count rate and an increase in the dissociation of caseins and the particle size because of increased repulsion. The number of succinylation sites for all casein proteins increased as the degree of succinylation increased from 10 to 44%, with the number of sites for  $\alpha_{s1}$ -CN,  $\alpha_{s2}$ -CN and  $\beta$ -CN increasing from 4 to 6, 2 to 5 and 2 to 7 respectively because the structure of casein made these residues easily accessible. At the higher degree of succinylation of 44%, succinylation occurred at Lys7, Lys34, Lys36, Lys42, Lys83 and Lys124 in  $\alpha_{s1}$ -CN, Lys80, Lys150, Lys152, Lys158 and Lys165 in  $\alpha_{s2}$ -CN, Lys28, Lys29, Lys32, Lys99, Lys105, Lys107 and Lys113 in  $\beta$ -CN and Lys75, Lys77, Lys83, Lys91, Lys100, Lys135 and Lys138 in  $\beta$ -lactoglobulin.

Succinylation of the milk was found to have a negative effect on the plasmin-induced hydrolysis of caseins, as observed in a previous study (Chapter 3), because of modification of the  $\epsilon$ -amino group of lysine into succinyl-lysine through succinylation, making it unrecognisable to the substrate-binding pocket of plasmin.

However, the trend was non-linear and different from that in the previous pure  $\beta$ -CN study; the decrease in the hydrolysis rate was linear at low degrees of succinylation but did not significantly decrease further with further increases in succinylation. This negative effect of succinylation on plasmin-induced hydrolysis correlates well with the micelle as a steric protector of the caseins from plasmin in terms of structural aspects. The majority of the plasmin-sensitive proteins are present in the micelle core. At lower degrees of succinylation, when there is little dissociation of the micelle, the decrease in the hydrolysis rate became linear because of the substrate modification; however at higher degrees of succinylation, it was the dissociation of the caseins from the micelle that dominated, resulting in an increase in the micelle mesh size (porosity), extensive unfolding and expansion of the polypeptide, which collectively reduced steric hindrance, making the plasmin-sensitive proteins readily available to undergo hydrolysis and the negative effect became less significant.

The present study gives more insight into the importance of the micellar structure in influencing hydrolysis. These results indicate that succinylation may be useful for understanding and controlling the plasmin-induced hydrolysis of milk proteins in terms of structural aspects of protection.

# Chapter 7: High-heat-induced changes in a milk system and their effect on plasmin-induced hydrolysis

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## 7.1 INTRODUCTION

Sterilisation and ultra-high-temperature (UHT) treatment are commonly used to increase the shelf life of milk and dairy beverages. However, plasminogen and its activators have been shown to be heat stable, surviving pasteurisation conditions (Metwalli et al., 1998; Richardson, 1983a; Saint Denis et al., 2001) and partially surviving UHT processes (Alichanidis et al., 1986; Driessen & van der Waals, 1978), which could lead to defects in the texture (gelation and sedimentation) and the taste (bitterness) of these products.

Such a high heat treatment of UHT milks and beverages to extend their shelf life could result in several physicochemical changes, such as protein conformational modifications, aggregations and Maillard reactions, in the milk system (van Boekel, 1998). Reactions occur at a relatively slow rate and are largely reversible at lower temperatures (< 90°C), whereas they occur rapidly and most are irreversible at higher temperatures (O'Connell & Fox, 2003). These heat-induced changes in milk systems can have an effect on plasmin-induced hydrolysis.

Following pasteurisation, the activity of plasmin increases, concomitant with a decrease in the plasminogen concentration, which is due to the denaturation of inhibitor(s) of plasminogen activator(s) (Richardson, 1983a). More severe heat treatment, i.e. UHT processing and sterilisation, causes a reduction in the plasmin-induced hydrolysis of casein because of the inactivation of plasmin by sulphhydryl–disulphide interchange reactions (Kennedy & Kelly, 1997), modification of the substrate by lactosylation (Bhatt et al., 2014) and/or physical inhibition through whey protein association with the casein micelles (Enright & Kelly, 1999).

Cysteine and/or  $\beta$ -lactoglobulin have been found to reduce plasmin activity through sulphhydryl–disulphide interchange reactions between the disulphide bridges of plasminogen and cysteine or  $\beta$ -lactoglobulin (Alichanidis et al., 1986; Grufferty & Fox, 1988c; Metwalli et al., 1998; Rollema & Poll, 1986; Ryan, Stevenson, & Hayes, 2012; Saint Denis et al., 2001) and the activation of plasminogen (Rollema & Poll, 1986; Saint Denis et al., 2001). The association of heat-denatured whey proteins with the casein micelles has been shown to cause a reduction in plasmin-induced hydrolysis by physically blocking plasmin from accessing the peptide bonds (Enright & Kelly, 1999). However, the effect on the plasmin-induced hydrolysis of milk of the association of whey proteins with the casein micelles along with lactosylation and other physicochemical changes that occur with high heat treatment has not been studied.

In the present study, the effect of heat-induced changes in milk caused by different components, e.g. whey proteins and lactose, along with the dissociation and association of proteins on plasmin-induced hydrolysis was investigated using a sequential approach. The whey proteins and the lactose were removed from milk and were added back sequentially to study the effect on plasmin-induced hydrolysis of each factor individually and in combination.

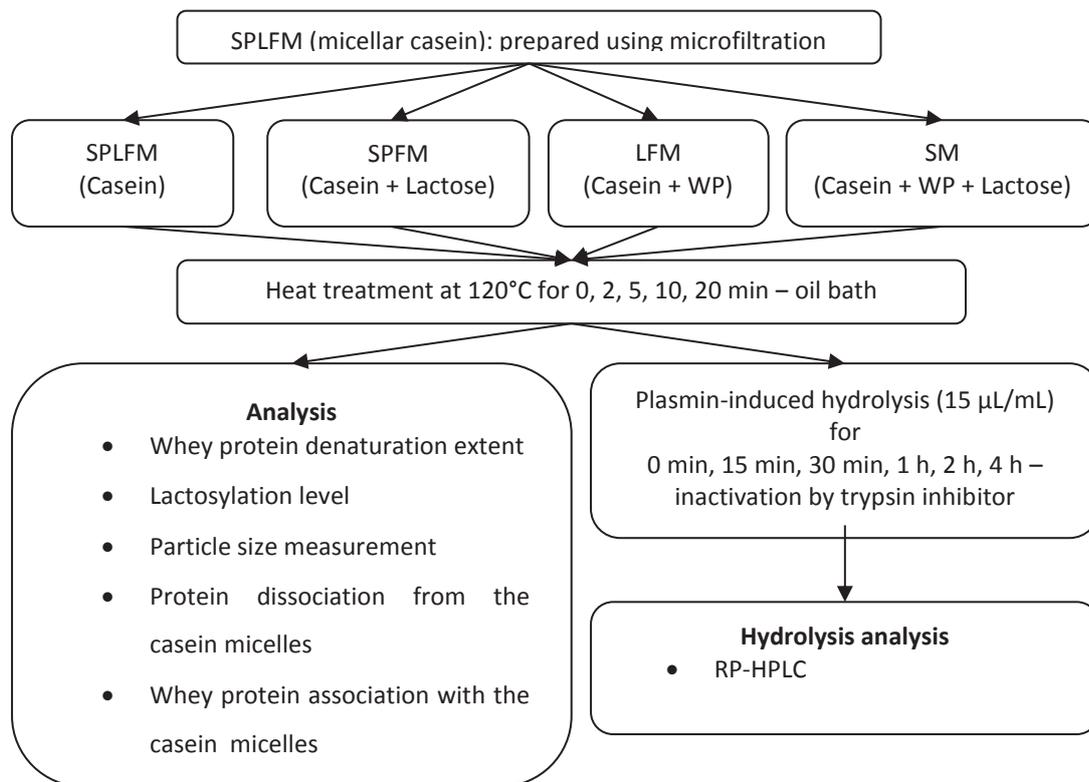
## 7.2 MATERIALS

Skim milk (pH 6.65) and whey protein isolate (WPI) were supplied by Fonterra Co-operative Group Ltd, Palmerston North, New Zealand. Plasmin from bovine plasma (5 U/mL) was purchased from Roche/Boehringer Mannheim GmbH (Germany). Chemicals for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and reverse-phase high performance liquid chromatography (RP-HPLC) buffers were purchased from Merck (Darmstadt, Germany). An acrylamide/bisacrylamide mixture 37.5:1 (2.6% C) from Bio-Rad (Hercules, CA, USA) was used. The different salts for making simulated milk ultrafiltrate (SMUF) were purchased from Merck (Germany). The water-free acetone used was from Thermo Fisher Scientific (Auckland, New Zealand). Fluorescamine (4-phenylspiro[furan-2(3H),19-phthalan]-3,39dione) was purchased from Sigma–Aldrich (Auckland, New Zealand).

## 7.3 METHODS

### 7.3.1 Preparation of various milk systems

A small quantity of sodium azide (0.01 g/100 g) was added to the skim milk as a preservative and different milk systems were prepared as shown in Scheme 3.



Scheme 3. Schematic diagram of the methodology.

### ***Preparation of serum-protein-free lactose-free milk***

Serum-protein-free lactose-free milk (SPLFM) was prepared by using a microfiltration technique. A skim milk sample was microfiltered using a membrane cartridge with a 0.1 µm pore size, which was sufficient to retain the casein in the retentate whereas the whey proteins (mainly  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin) and lactose passed through into the permeate/microfiltrate. To achieve micellar casein, the salt balance was maintained by the addition of SMUF to the milk feed at the same rate as the microfiltrate flow rate. The same procedures/cycles were repeated until less than 5% of the original  $\beta$ -lactoglobulin was achieved.

Table 10. Description of the various milk systems

Short form	What is it?	Preparation
SPLFM	Serum-protein-free lactose-free milk	Micellar casein prepared using microfiltration
SPFM	Serum-protein-free milk	Micellar casein + lactose
LFM	Lactose-free milk	Micellar casein + whey protein isolate
SM	Skim milk	Micellar casein + whey protein isolate + lactose

SMUF was prepared by adding different salts in a sequential order, with continuous stirring, as described in Table 11. Sodium azide was added as a preservative and potassium hydroxide was added to adjust the pH to 6.6.

Table 11. Preparation of lactose-free SMUF based on method from Jenness and Koops (1962).

Chemicals	Molecular weight (g/mol)	Weight (g)	Moles
$\text{KH}_2\text{PO}_4$	136.09	1.58	0.0116
$\text{K}_3\text{Citrate}\cdot\text{H}_2\text{O}$	324.42	0.5	0.00154
$\text{Na}_3\text{Citrate}\cdot 2\text{H}_2\text{O}$	294.16	1.794	0.00610
$\text{K}_2\text{SO}_4$	174.87	0.18	0.0010
$\text{CaCl}_2\cdot 2\text{H}_2\text{O}$	147.02	1.32	0.0090
$\text{MgCl}_2\cdot 6\text{H}_2\text{O}$	203.3	0.244	0.0012
MgHCitrate	286	0.572	0.0020
$\text{K}_2\text{CO}_3$	138.21	0.3	0.0022
KCl	74.56	0.977	0.0131
Water	18	1000	55.56
Total		1007.857	

### ***Addition of WPI to achieve lactose-free milk (LFM)***

To return the whey protein to obtain LFM, WPI was added into SPLFM to achieve a ratio of casein to whey protein similar to that in the starting skim milk.

Table 12. Protein comparison after reconstitution using SDS-PAGE

Protein	SPLFM + WPI	Skim milk
$\alpha$ -Casein	40.9	41.0
$\beta$ -Casein	27.2	29.8
$\kappa$ -Casein	10.6	9.8
$\beta$ -Lactoglobulin	17.2	15.6
$\alpha$ -Lactalbumin	4.0	4.8

### ***Addition of lactose to achieve serum-protein-free milk (SPFM)***

Lactose was added back to SPLFM to achieve a lactose content of 4.8% in the final SPFM.

### ***Addition of lactose and WPI to achieve a skim-milk-like system (SM)***

Lactose and WPI were added to SPLFM to achieve a skim-milk-like system containing 4.8% lactose and similar protein ratios (Table 12).

### **7.3.2 Adjustment of pH**

The pH values of the different milk systems were adjusted to pH 6.6 before and after heat treatment by the slow addition of 1 M HCl or 1 M NaOH to well-stirred solutions at room temperature, to achieve the same pH as that of the original skim milk. The milk samples were allowed to equilibrate for 2 h before the final pH reading and minor readjustment.

### **7.3.3 Heat treatment**

The milk samples were equilibrated at 30°C before heat treatment. Subsamples (5 mL) from all the different milk systems (SPLFM, SPFM, LFM and SM) were transferred to glass vials and heated at 120°C for 0, 2, 5, 10 and 20 min, with an additional 1 min of come-up time. The high heat treatment was chosen to achieve both lactosylation and whey protein association with the casein micelle at various levels by varying heating time. Heating temperature of 120°C was closest to the retort sterilisation temperature and most feasible at a lab scale set-up. The heat treatment was applied in a thermostatically controlled oil bath with a continuous rocking assembly. After the heat treatment, the samples were cooled at room

temperature by keeping the vials in cold running water (~ 15°C) and were kept for 24 h to re-equilibrate the pH.

#### **7.3.4 Measurement of level of modification/lactosylation by the fluorescamine method**

The decrease in the number of reactive amino groups on the protein that is caused by heat-induced lactosylation can be used as a measure of the level of lactosylation and was measured using the fluorescamine method, as described previously (Bhatt et al., 2014). All measurements were carried out in triplicate.

#### **7.3.5 Particle size measurement**

Particle size measurements were carried out on the different milk samples to observe the effect of heat treatment on the casein micelle size distribution in the different systems. The particle size was measured by photon correlation spectroscopy using a Malvern Zetasizer 4 instrument and the associated ZET5110 particle sizing cell (Malvern Instruments Ltd, Malvern, Worcs., UK), by the method of Anema and Li (2003a). Milk samples (50 µL) were dispersed in 5 mL of SMUF before particle size measurement.

#### **7.3.6 Extent of whey protein denaturation**

The extent of whey protein denaturation was measured using RP-HPLC (Elgar et al., 2000). pH-4.6-soluble fractions of the different milk systems were prepared by lowering the pH to pH 4.6 with 0.5 M sodium acetate buffer, and allowing the mixtures to stand for 1 h at room temperature (~ 20°C). The mixtures were centrifuged at 14,000 rev/min (21,000 g) for 15 min and the supernatants were collected to yield the pH 4.6 filtrates.

The method of Elgar et al. (2000) was used for the RP-HPLC analysis. The HPLC system consisted of a Waters 2690 Alliance Separation Module (Waters, Milford, MA, USA) interfaced with a Waters 486 MS tunable absorbance detector and a Waters Millennium 32 data acquisition and manipulation system. Queued samples were refrigerated at 5°C. The extent of whey protein denaturation was calculated with reference to control (un-heated) samples. It was measured as the decrease in the concentration of the whey proteins in the pH-4.6-soluble fraction upon heat

treatment.  $\beta$ -Lactoglobulin and  $\alpha$ -lactalbumin were quantified and their total was referred to as total whey protein.

### **7.3.7 Centrifugation**

Centrifugation was used to identify the extent of casein dissociation from the micelle into the serum phase and to identify the association of whey proteins with the casein micelles. The whey protein content of the supernatant after centrifuging out the casein micelles and associated whey proteins was measured (Oldfield, Singh, & Taylor, 1998; Oldfield, Singh, Taylor, & Pearce, 2000).

The centrifugation was done using the method of Anema (2007). One millilitre of milk sample was transferred into an Eppendorf tube (total volume 1.5 mL) and was centrifuged at 14,000 rev/min ( $\sim 21,000 g$ ) for 1 h at 20°C in an Eppendorf centrifuge Type 5417C (Eppendorf AG, Hamburg, Germany); the non-sedimentable caseins and whey proteins were defined as serum-phase caseins and whey proteins respectively (Anema, 2007). The depletion of the different caseins from the micelle and the levels of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin in the serum were measured using SDS-PAGE and quantifying the different proteins.

### **7.3.8 SDS-PAGE**

To study the effect of heat treatment on the dissociation of the casein micelle and the attachment of whey proteins to the casein micelle, SDS-PAGE was performed under reducing conditions as described by Anema and Klostermeyer (1997).

Different samples were dispersed in SDS sample buffer [0.5 M Tris-HCl buffer, pH 6.6, containing 2% (w/v) SDS] in a 1:30 ratio (v/v), and 10  $\mu$ L of prepared sample was loaded per SDS-PAGE well. The gels were run at 210 V and 70 mA for approximately 1 h. After electrophoresis, the gels were stained using a staining solution [0.1% (w/v) Amido Black 10B in 10% (v/v) acetic acid and 25% (v/v) isopropanol] for 1 h and then destained using 10% (v/v) acetic acid solution until a clear background was achieved. The gels were scanned (GE Image Scanner III, GE Healthcare Ltd, New Zealand) and the protein bands on the gel were quantified

using ImageQuant TL (v 2005) software to give a numerical protein–dye absorbance value.

### **7.3.9 Plasmin-induced hydrolysis**

The different milk samples were hydrolysed by adding plasmin at 15  $\mu\text{L}/\text{mL}$  (0.1 U/mL) of milk. The added plasmin concentration was more than 1000 times higher than naturally present in milk to facilitate rapid hydrolysis for the study and minimise other side reactions. The samples were incubated in a water bath at 37°C and were allowed to equilibrate to temperature for 1 h before plasmin addition; the hydrolysis pattern was observed at incubation times of 0 min, 15 min, 30 min, 1 h, 2 h and 4 h. The samples were removed from the water bath after each incubation time and the reaction was stopped immediately by adding 50  $\mu\text{L}$  of SDS (2%).

### **7.3.10 RP-HPLC**

To measure the extent of hydrolysis using RP-HPLC, pH-4.6-soluble fractions were prepared by the same method as that used in measuring the extent of whey protein denaturation.

HPLC analysis was performed on Agilent 1100 series equipment with a UV/VIS photodiode array multi-wavelength detector using a 250 mm x 4.6 mm reversed-phase column (Phenomenex-Jupiter 300 C18, 5  $\mu\text{m}$ ) at 40°C and a binary solvent gradient system at a flow rate of 1 mL/min and detection at 210 nm. The solvents used were solvent A [0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water] and solvent B [0.1% (v/v) TFA in HPLC-grade acetonitrile]. The proportion of solvent B was increased from 15 to 35% during the first 20 min and, after a further 5 min, increased to 65% in 35 min and then, after a further 5 min, finally reduced back to 15% solvent B over a 5 min period, in readiness for the next sample injection. Injections of 50  $\mu\text{L}$  of filtrate were made by auto-injector. All samples were analysed in duplicate and data analysis was performed using Agilent (Hewlett Packard) Chemstation software. The peptides were identified as proteose peptones (PP5, PP8slow and PP8fast) and were quantified by integration of peak areas.

All experiments reported were repeated at least twice with the same milk samples. In addition, the experiments were repeated with several different milk samples. Although there were some variations between individual milks, the same trends and relationships as reported here were found for all samples examined to date.

## 7.4 RESULTS AND DISCUSSION

### 7.4.1 Heat-induced changes in the systems

#### *Extent of whey protein denaturation*

Whey proteins, e.g.  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin, are globular proteins and retain their native conformation within limited pH and temperature ranges (Anema & McKenna, 1996). Whey proteins are relatively heat labile compared with caseins and, when milk is heated above 70°C, the whey proteins  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin unfold and irreversibly denature (Dalglish, 1990; Relkin & Mulvihill, 1996). Denaturation of the whey proteins results in considerable changes in the native conformation; the three-dimensional (tertiary) structure of the polypeptide chain is converted to a lower state of order (Anema & McKenna, 1996).

The extent of whey protein denaturation was measured using RP-HPLC. The whey proteins were readily denatured at 120°C and, after 2 min of heat treatment, more than 85% of  $\beta$ -lactoglobulin was denatured in both the presence and the absence of lactose (Figure 51A). With an increase in the heating time, the extent of denaturation increased further.

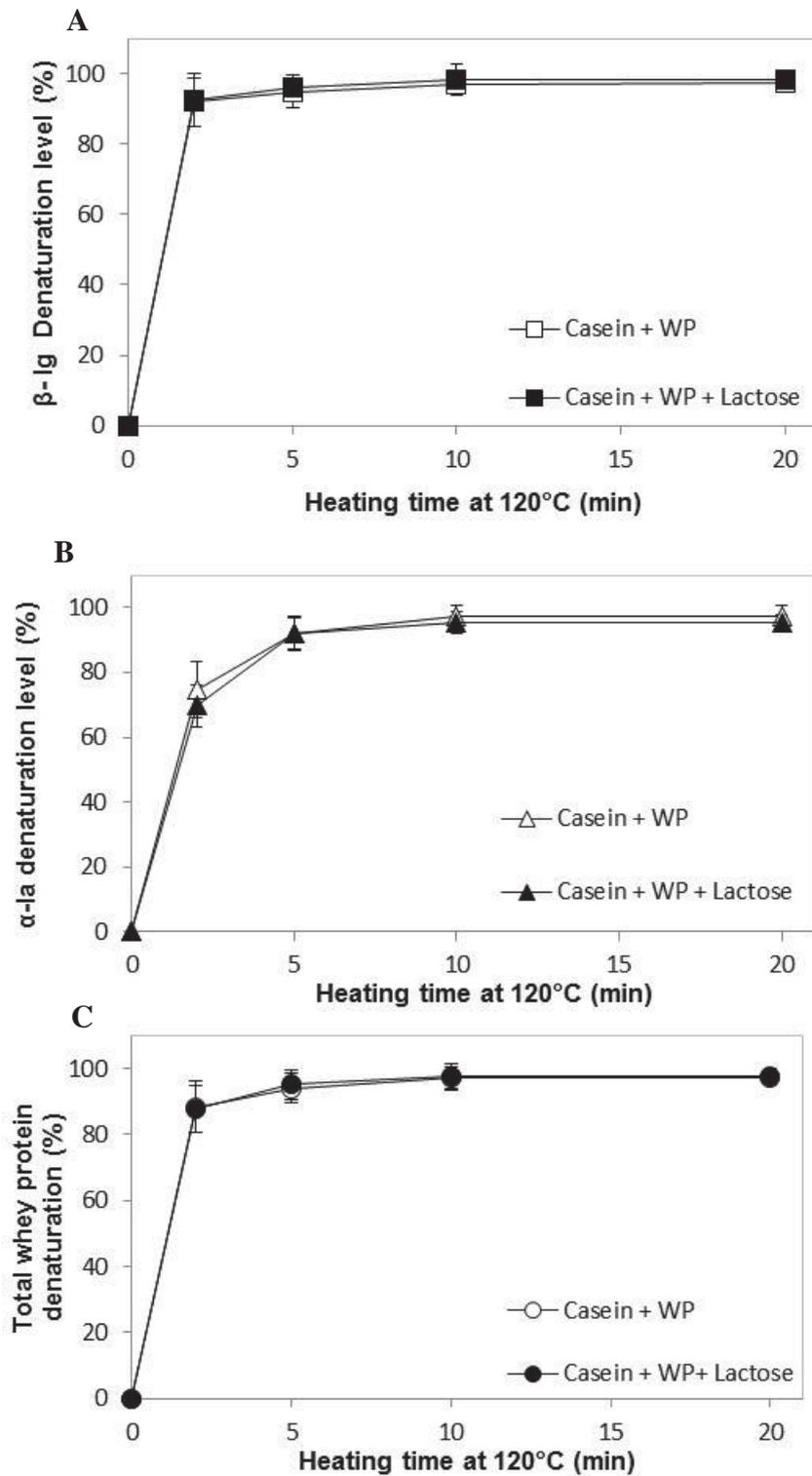


Figure 51. Effect of heat treatment on extent of whey protein denaturation: A,  $\beta$ -lactoglobulin; B,  $\alpha$ -lactalbumin; C, total whey protein. Error bars refer to standard deviations.

Among the different whey proteins, the immunoglobulins are the least heat resistant and  $\alpha$ -lactalbumin is the most heat resistant, with  $\beta$ -lactoglobulin and the

serum albumins having intermediate sensitivity (Larson & Roller, 1955; Lyster, 1970). The rate of denaturation of  $\alpha$ -lactalbumin was relatively slower than that of  $\beta$ -lactoglobulin; after 2 min of heat treatment, more than 75% was denatured (Figure 51B). With an increase in the heating time, the extent of denaturation increased further and it took 5 min at 120°C for  $\alpha$ -lactalbumin to achieve a similar extent of denaturation to that of  $\beta$ -lactoglobulin, which may have mainly been due to the difference in the reaction orders. These results were in agreement with the observations of Oldfield, Singh, and Taylor (2005). Reaction orders of 1.5 and 1 have been reported for the denaturations of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin respectively (Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Oldfield et al., 2005). Dannenberg and Kessler (1988) also observed abrupt changes in the temperature dependence of the rate constants ( $\beta$ -lactoglobulin at 90°C,  $\alpha$ -lactalbumin at 80°C) and attributed this to the different activation energies and entropies in the two temperature ranges.

Most of the whey proteins were denatured after 10 min of heat treatment. For the extent of denaturation of the total whey protein, the presence or the absence of lactose was not found to have a major effect (Figure 51C). The thermal denaturation of  $\beta$ -lactoglobulin is affected by calcium ions, lactose, pH, casein and the whey protein concentration (O'Connell & Fox, 2001). Although sugar has been found to have a protective action against the heat denaturation of proteins (Bull & Breese, 1978; de Wit & Klarenbeek, 1981; Itoh, Wada, & Nakanishi, 1976), we did not observe any changes in the extent of denaturation between lactose-free milk and milk with lactose. This could have been because the heat treatment used was high enough to cause instantaneous denaturation, thus not allowing a timescale in which to observe the differences, or alternatively because, under the conditions used in this experiment, there was no difference.

#### ***Association of whey proteins with the casein micelles***

Denaturation and unfolding of the whey proteins upon heat treatment exposes hydrophobic amino acid residues that were previously buried deep inside the native protein structure and results in an increase in the reactivity of such groups (Singh & Havea, 2003). There is also an increase in the reactivity of the sulphhydryl groups of

cysteine, which are involved in disulphide interchange reactions at higher temperatures (Jang & Swaisgood, 1990); however, an unfolded protein can be more susceptible to protein–protein interactions via calcium bridging and hydrophobic bonding (Anema & McKenna, 1996; Singh & Havea, 2003). At lower temperatures (~70°C), hydrophobic bonding is principally responsible for the complex formation between  $\kappa$ -casein and  $\beta$ -lactoglobulin (Haque & Kinsella, 1988; Jang & Swaisgood, 1990); however, at higher temperatures, sulphhydryl–disulphide interchange reactions are involved (Jang & Swaisgood, 1990; Sawyer, 1969).

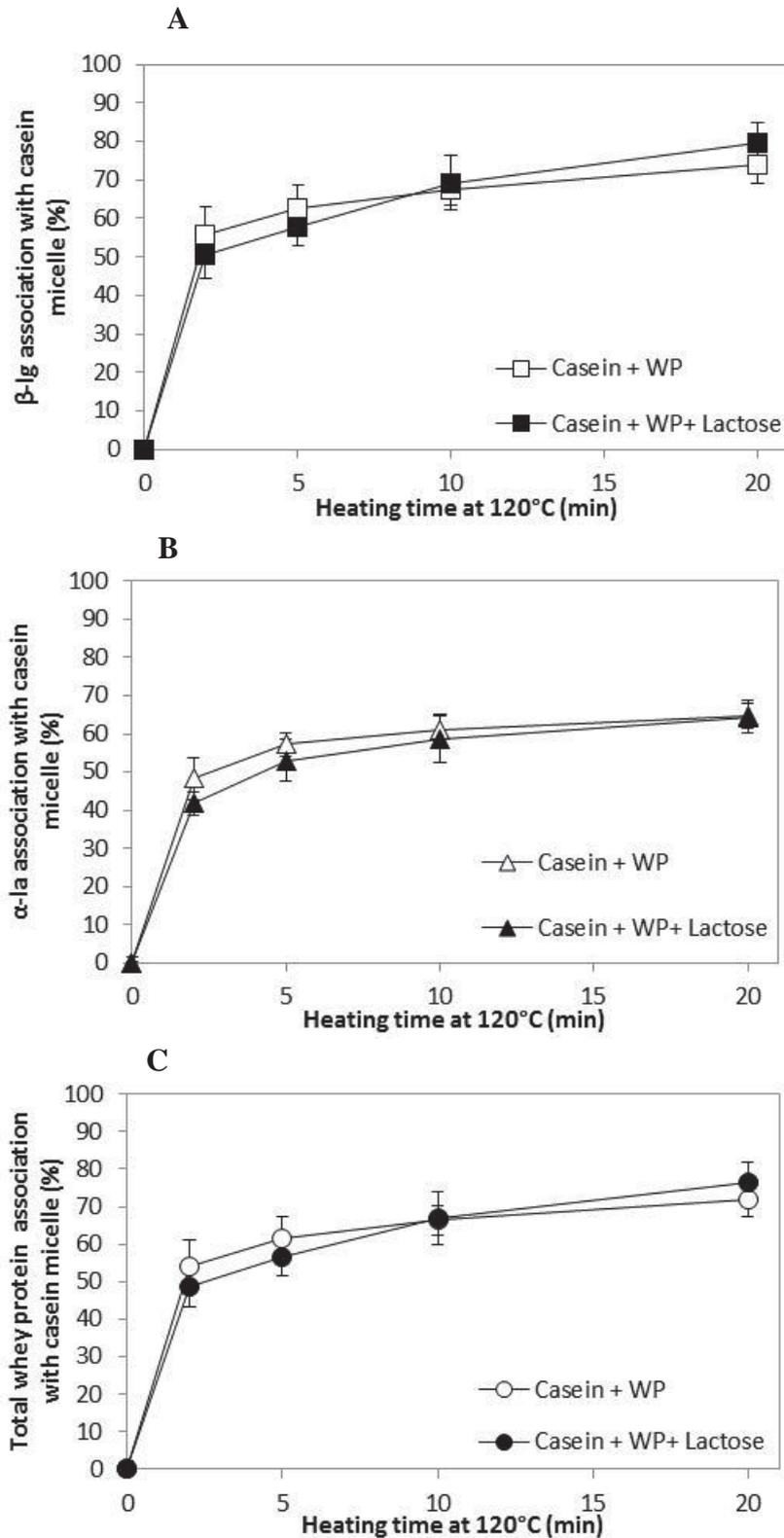


Figure 52. Effect of heat treatment on the association of the whey proteins with the casein micelles: A,  $\beta$ -lactoglobulin; B,  $\alpha$ -lactalbumin; C, total whey protein. Error bars refer to standard deviations.

The denatured whey proteins bind to the casein micelles to form whey protein- $\kappa$ -casein complexes (Guyomarc'h, Law, & Dalgleish, 2003; Jang & Swaisgood, 1990;

Singh & Fox, 1987a) preferentially (Donato, Guyomarc'h, Amiot, & Dalgleish, 2007), and are seen as appendages or protuberances (Creamer, Berry, & Matheson, 1978; Mohammad & Fox, 1987; Singh, Sharma, Taylor, & Creamer, 1996), or they may simply self-aggregate to form polymeric products (Dalgleish, 1990). Dalgleish (1990) observed a direct relationship between the formation of aggregates and the amount of serum protein lost.

With an increase in the heating time, the association of the whey proteins with the casein micelles increased in both milk systems: casein + WPI and casein + WPI + lactose. After 20 min of heating time at approximately 120°C, 80% of the whey proteins were associated with the casein micelles in both milk systems, and there was not much difference in the association behaviour of the systems.

Figure 51 and Figure 52 clearly show that the rate of whey protein denaturation was much higher than the rate of whey protein association with the casein micelles, which is in agreement with previous observations (Anema & Li, 2003a; Oldfield et al., 1998). The association with the casein micelles was greater for  $\beta$ -lactoglobulin (Figure 52A) than for  $\alpha$ -lactalbumin (Figure 52B) throughout the heating time. Oldfield et al. (1998) reported a similar observation in the 80–130°C heating range. The differences in association behaviour could have been due to the slower rate of denaturation of  $\alpha$ -lactalbumin, compared with  $\beta$ -lactoglobulin, as discussed above. According to Corredig and Dalgleish (1999),  $\beta$ -lactoglobulin must be present for there to be an association of the whey proteins with the casein micelles. This could be either because of indirect interactions of  $\alpha$ -lactalbumin, in contrast to  $\beta$ -lactoglobulin, with the casein micelles (Noh, Richardson, & Creamer, 1989; Smits & van Brouwershaven, 1980), occurring through the formation of an intermediate between  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin in the serum phase followed by its interaction with  $\kappa$ -casein on the micelle (Corredig & Dalgleish, 1999), or because  $\alpha$ -lactalbumin interacts with  $\beta$ -lactoglobulin– $\kappa$ -casein complexes at the micelle surface (Fairise, Cayot, & Lorient, 1999).

According to previous studies, the distribution of  $\beta$ -lactoglobulin– $\kappa$ -casein complexes between the serum phase and the colloidal phase is pH dependent

(Anema & Li, 2003b; Oldfield et al., 2000; Singh et al., 1996); complexes are present in the colloidal phase, via attachment to the casein micelle, at lower pH (< 6.6) and in the serum phase at higher pH (> 6.6) (Vasbinder & de Kruif, 2003). Therefore, to achieve maximum association of the whey proteins with the casein micelles in the present study, the pH of all milk systems was adjusted to 6.6. In the present study, SMUF was used during microfiltration, to achieve intact casein micelles without disturbing the natural ionic strength of the system; the ionic strength of the system is known to affect interactions between  $\beta$ -lactoglobulin and  $\kappa$ -casein (El-Negoumy, 1974).

### **Particle size**

The effect of heat treatment at 120°C on the particle size of the different milk systems is shown in Figure 53. There was an increase in the casein micelle size of about 45–55 nm after 20 min of heat treatment in the systems containing whey proteins.

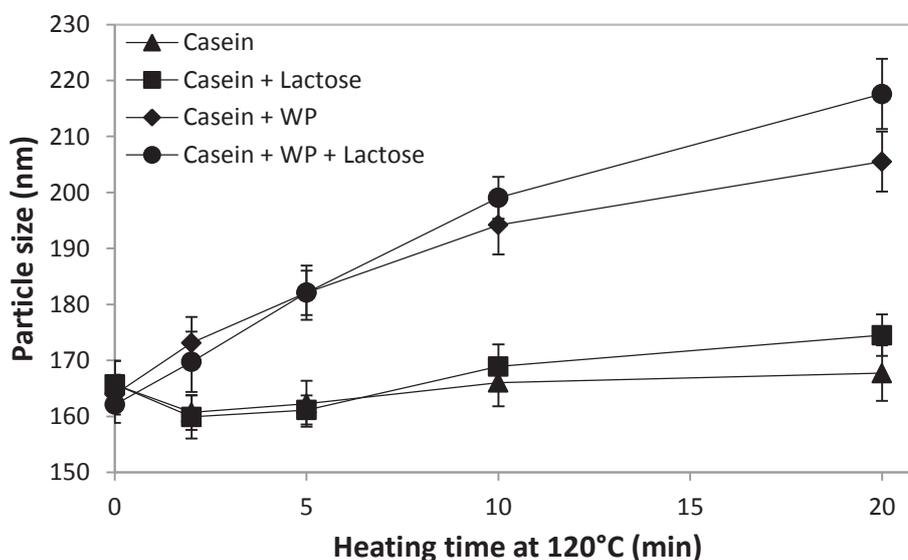


Figure 53. Effect of high heat treatment on the change in particle size of the different milk systems (as measured by dynamic light scattering).

With increasing heat treatment, the particle size increased linearly for LFM ( $R^2 = 0.9317$ ) and SM ( $R^2 = 0.9673$ ). In contrast, this was not the case for SPFM and SPLFM, suggesting that the role of whey proteins in increasing the particle size is in agreement with Anema and Li (2003a). As discussed previously, the formation of

appendages or protuberances (Creamer et al., 1978; Mohammad & Fox, 1987; Singh et al., 1996), because of the formation of whey protein- $\kappa$ -casein complexes on the micelle, could have been the main reason for the increase in particle size.

The casein micelle size was slightly higher in SM samples than in LFM samples, which could have been due to Maillard-reaction-induced cross-linking in the SM keeping the whey protein- $\kappa$ -casein complexes more attached to the casein micelles than in LFM samples.

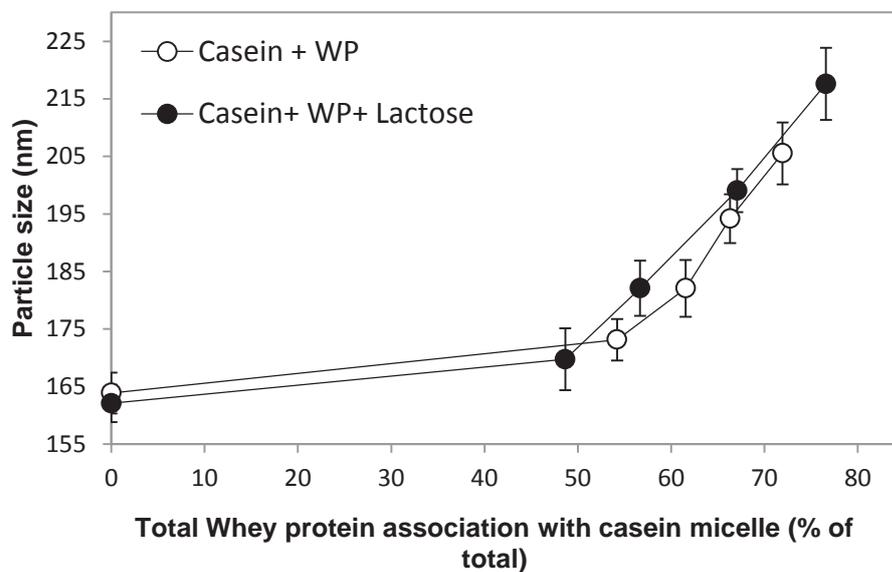


Figure 54. Effect of whey protein association with the casein micelles on particle size.

The association of the whey proteins with the casein micelles correlated well with the particle size of the casein micelle (Figure 54) and correlated poorly with the extent of whey protein denaturation (Figure 51); these observations at higher heating temperature (120°C) are in agreement with the observations of Anema and Li (2003a) at lower heating temperatures (< 100°C).

For the first 2 min of heat treatment, the particle size for SPFM and SPLFM decreased, with a greater decrease for SPLFM. Anema and Li (2003a) also observed a small (~ 5 nm) decrease in casein micelle size at very short heating times before a gradual increase, in agreement with the observations of Jeurnink (1992) and Jeurnink and de Kruif (1993) based on turbidity and viscosity measurements of heated milk samples; they attributed this to shrinkage of the casein micelles

because of the precipitation of calcium phosphate on to the micelles. With further increases in the heat treatment, the particle size of SPFM and SPLFM began to increase and became similar to that of the unheated milk after 10 min of heat treatment. It continued to increase slightly (~ 5 nm) for SPFM whereas there was not much increase in size for SPLFM after 20 min, compared with the initial particle size. Such a slight increase could have been because of the residual presence of whey proteins (less than 5% of the total whey protein) in these milks.

### ***Dissociation of caseins from the casein micelles***

Dissociation of protein from the micelle is known to occur (Anema, 1998; Aoki, Suzuki, & Imamura, 1974, 1975; Fox, Harper, Holsinger, & Pallansch, 1967) during high heat treatment. Such dissociation of caseins from the micelle could make them more prone to enzyme action because of ease of accessibility.

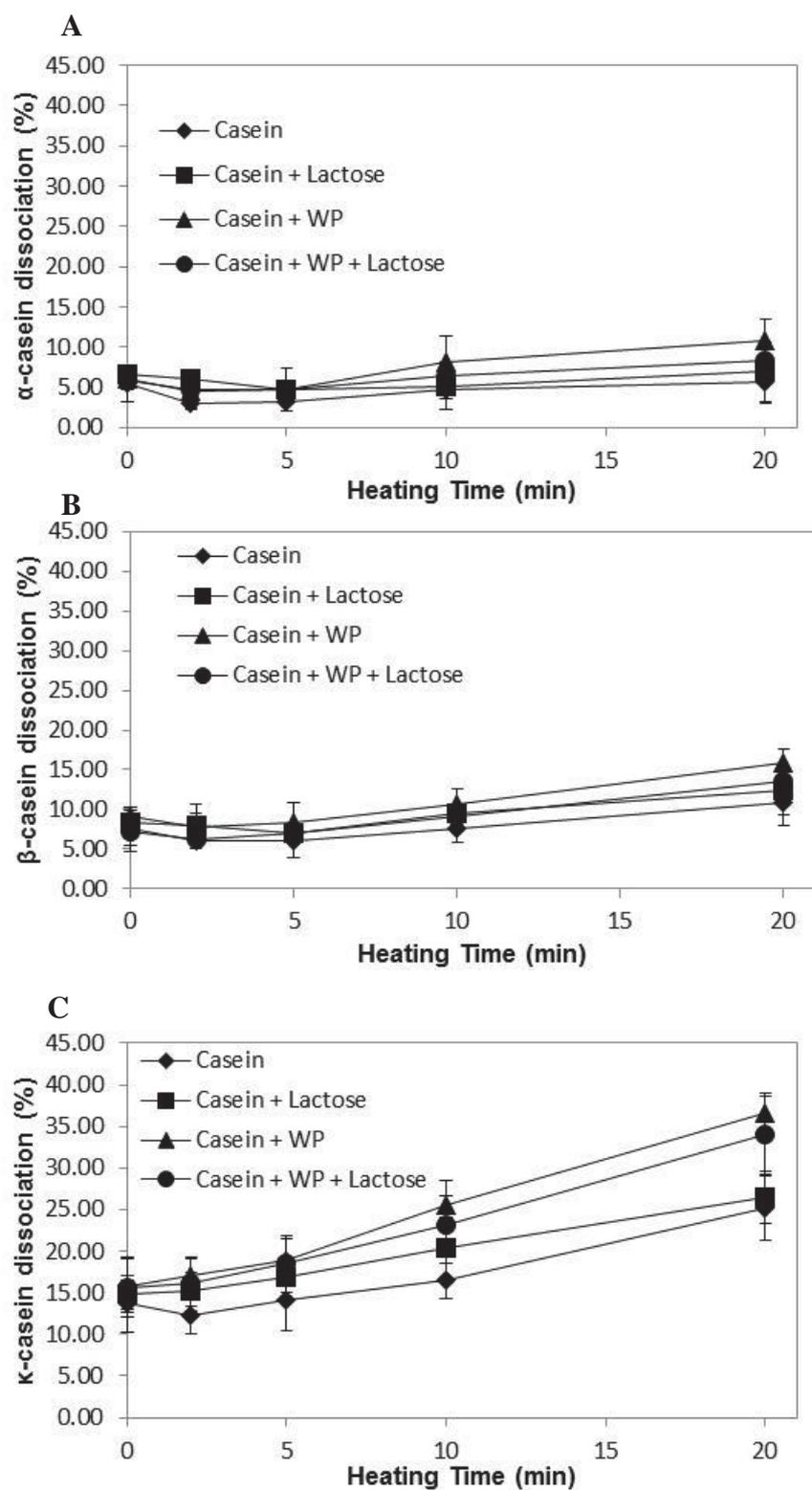


Figure 55. Effect of high heat treatment on the extent of protein dissociation from the casein micelles as measured in the serum phase. Error bars refer to standard deviations.

The dissociation of proteins from the casein micelles increased with an increase in the heat treatment (Figure 55), in agreement with the previous observations of Fox

et al. (1967) in milk and Aoki et al. (1974, 1975) in serum-protein-free casein micelle (SPFCM) dispersions when heated at temperatures  $> 110^{\circ}\text{C}$ . Figure 55 shows that  $\kappa$ -casein dissociated most from the micelles into the serum phase upon heat treatment, whereas there was only slight dissociation of  $\alpha_s$ -casein (6–10%) and  $\beta$ -casein (9–15%). It is known that  $\kappa$ -casein is not the main target and is resistant to plasmin's action (Bastian & Brown, 1996); therefore, the increase in its accessibility to plasmin because of its presence in the serum phase will not have an impact on the overall hydrolysis rate. The dissociation of  $\kappa$ -casein from the micelles alters the surface of the micelles; the surface charge and the steric repulsions decrease because of the loss of  $\kappa$ -casein (Singh & Latham, 1993); furthermore, the dissociation of  $\kappa$ -casein will expose the core of the casein micelle and the slight increase in the dissociation of  $\alpha_s$ -casein and  $\beta$ -casein from the micelle could increase the accessibility of these proteins to various reactions along with the hydrolysis.

It was observed that the casein-only milk (SPLFM) had less dissociation of  $\kappa$ -casein; when whey proteins were added, the dissociation was increased in LFM. There have been similar observations at pH 6.7–7.1; greater dissociation of the  $\kappa$ -casein than that found in whey-protein-free milk was observed upon the heat treatment of skim milk or of whey-protein-free milk to which increased concentrations of whey protein were added (Anema & Li, 2000; Singh & Fox, 1987b).

It was also observed that there was less dissociation of  $\kappa$ -casein in the presence of lactose than in LFM. The increase in the dissociation of proteins from the micelles with heat treatment is attributed to the solubilisation of micellar calcium (Aoki et al., 1975), to the weakening of micellar protein–protein interactions as a result of charge modification because of lactosylation (Singh & Creamer, 1991a, 1991b, 1991c) and to dephosphorylation (Dalgleish, Pouliot, & Paquin, 1987).

### ***Lactosylation***

Heat treatment of the system results in the attachment of lactose to the  $\epsilon$ -amino groups of the lysine residues of milk proteins, leading to the formation of the stable and dominant intermediate lactulosyl-lysine (the Amadori rearrangement product);

the reaction is known as lactosylation (Léonil et al., 1997; van Boekel, 1998). Heat-induced lactosylation was determined by measuring the relative number of reactive amino groups using the fluorescamine method (Bhatt et al., 2014).

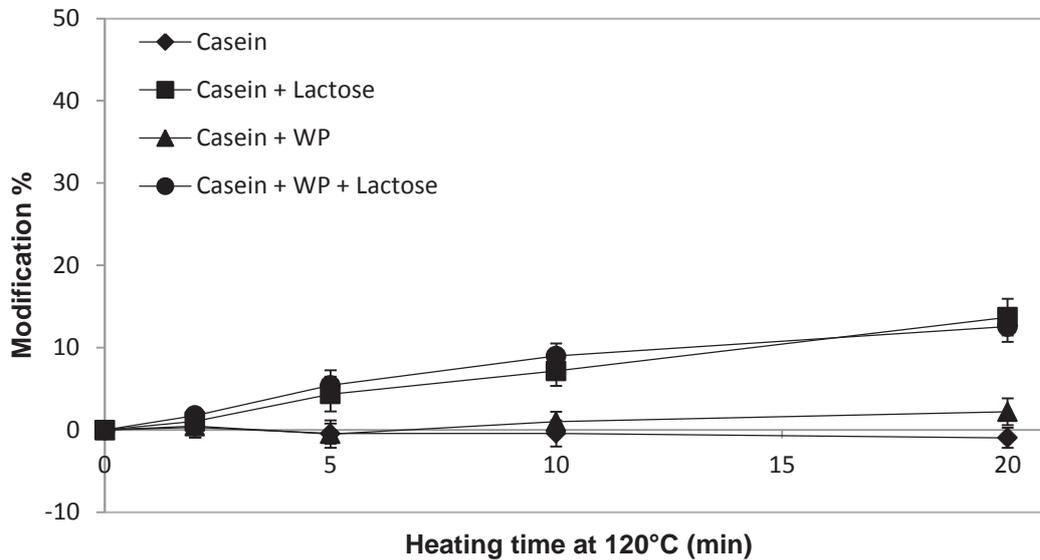


Figure 56. Effect of high heat treatment on the level of lactosylation (as measured by the change in the number of free amino groups on the proteins by the fluorescamine method). Error bars refer to standard deviations.

Lactosylation in the presence of lactose was found to increase linearly (Figure 56) with an increase in the heating time at 120°C; similar results were observed by Bhatt et al. (2014) in a  $\beta$ -casein model system. The presence and/or the absence of whey proteins was not found to have much impact on the level of lactosylation, which could have been due to lactosylation of the whey proteins in SM (casein + WPI + lactose) samples. The absence of lactose was not found to have much effect on the loss of free amino groups except in the LFM (casein + WPI) sample heated for 20 min, with around 2% decrease in free amino groups.

Caseins, i.e.  $\alpha_{s1}$ -casein and  $\beta$ -casein, being unstructured and abundant sources of accessible  $\epsilon$ -amino groups, are the main targets of the lactosylation in milk (Scaloni et al., 2002; Venkatachalam et al., 1993). Scaloni et al. (2002) identified the lactosylation sites in milk using a combined matrix-assisted laser desorption ionisation–mass spectrometry/Edman degradation approach on enzymatic digests. In moderately heat-treated milks, Scaloni et al. (2002) observed that lactosylation

occurred specifically at Lys34 ( $\alpha_{s1}$ -casein) and Lys107 ( $\beta$ -casein); however, samples subjected to more severe conditions were modified at Lys7, Lys34, Lys83, Lys103, Lys105, Lys132 and Lys193 ( $\alpha_{s1}$ -casein) and at Lys32, Lys48, Lys107, Lys113 and Lys176 ( $\beta$ -casein). The lactosylation of caseins at plasmin-targeted lysine residues could affect plasmin-induced hydrolysis, as observed in previous studies on the lactosylation (Bhatt et al., 2014; Dalsgaard et al., 2007) and succinylation (Chapter 3) of pure protein model systems.

#### 7.4.2 Effect of heat-induced changes on plasmin-induced hydrolysis

To examine the effects of whey protein association and lactosylation separately and in combination, whey proteins and lactose were removed from the skim milk using microfiltration and were added back to generate SPLFM, LFM, SPFM and SM.

With an increase in the heating time at 120°C, plasmin-induced hydrolysis was found to decrease linearly in all samples except SPLFM, suggesting that whey proteins and lactose have a role in the action against plasmin (Figure 57).

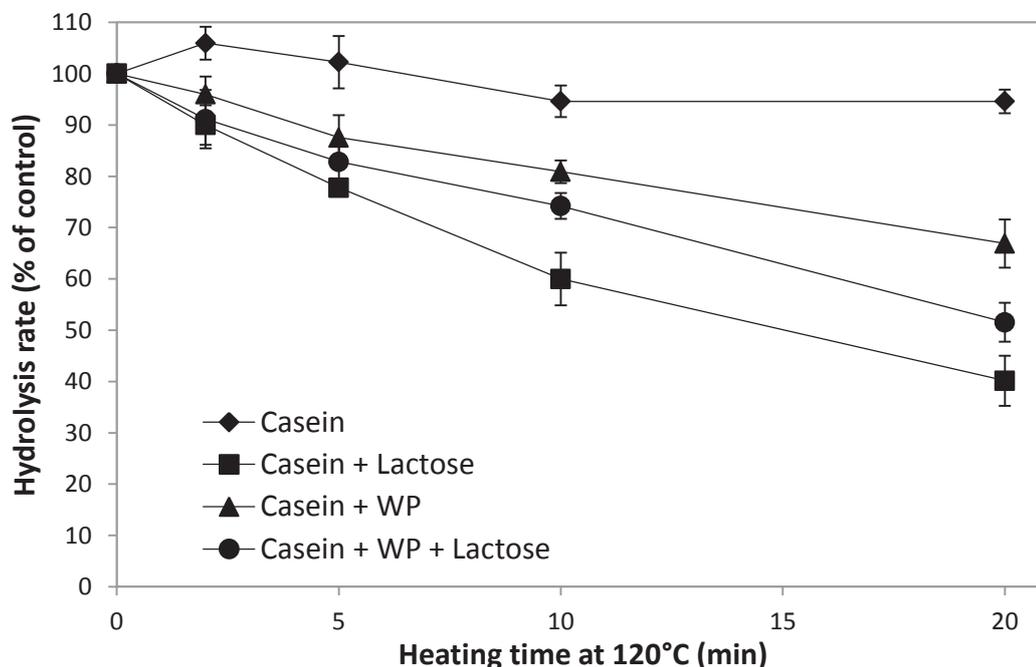


Figure 57. Effect of high heat treatment on the rate of plasmin-induced hydrolysis (as measured by the generation of peptides using RP-HPLC). Error bars refer to standard deviations.

The negative effect of lactosylation on plasmin-induced hydrolysis has already been shown in pure protein model systems (Bhatt et al., 2014; Dalsgaard et al., 2007). Similar results were observed in the present study in real milk systems, in which the caseins are in the casein micelles, suggesting that lactosylated proteins are resistant to plasmin-induced hydrolysis, independent of whether it is in simple non-micellar (open) structures or complex micellar structures.

LFM (casein + WPI) showed a decrease in plasmin hydrolysis, which may have been due mainly to an increase in the association of whey proteins with the casein micelles (Figure 52 and Figure 54). The linear decrease in plasmin-induced hydrolysis in SM (casein + lactose + WPI) could have been due to both the association of whey proteins (Figure 52) and the lactosylation of caseins (Figure 56). The negative effect on plasmin-induced hydrolysis was intermediate in SM (greater than in LFM but less than in SPFM) (Figure 57), which could have been due to a distributive effect of lactosylation, in which whey proteins are also lactosylated but are not the substrate for the action of plasmin. High heat treatment of SPLFM (casein) did not affect plasmin-induced hydrolysis, which could have been due to the absence of lactose and whey proteins.

When the results of the various treatments were compared, they indicated that SPFM showed the maximum decrease in the plasmin hydrolysis rate, followed by SM and LFM (Figure 57). The observed maximum negative effect on plasmin-induced hydrolysis in SPFM (casein + lactose) followed by SM was well correlated with the linear increase in lactosylation with heating time (Figure 56). From the observations, it can be concluded that lactosylation had a greater negative impact on plasmin-induced hydrolysis than the association of whey proteins with the casein micelles (protective effect).

The resistance to plasmin of high-heat-treated milk is explained by the schematic in Figure 58. High heat treatment in the presence of lactose causes lactosylation (the formation of lactulosyl-lysine), making the protein unrecognisable to plasmin's specificity pocket (Bhatt et al., 2014) and thus resulting in a decrease in hydrolysis in SPFM (casein + lactose) and SM (casein + WPI + lactose) samples.

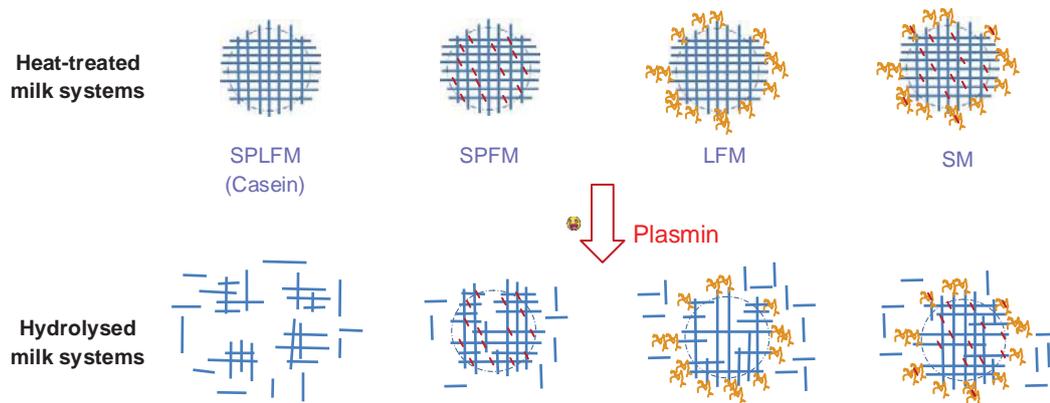


Figure 58. A simplistic mesh diagrammatic representation of the mechanism for the plasmin resistance of high-heat-treated milk: mesh lines, casein chains; red lines, lactosylation sites; yellow lines, denatured whey proteins.

Whey proteins, when attached to the casein micelle, are known to have a steric hindrance effect on the accessibility of the susceptible bonds of casein in the casein micelle (Benfeldt, Sørensen, Ellegård, & Petersen, 1997; Enright & Kelly, 1999; Lau, Barbano, & Rasmussen, 1991). According to Lau et al. (1991), heat-induced interactions of the whey proteins with the casein micelles, caused by pasteurisation, may change the accessibility of caseins to proteolytic enzymes and may cause differences in proteolysis during cheese aging. On the heat treatment of LFM and SM (Figure 58), whey proteins associated with the casein micelles (Figure 52 and Figure 53), such that the covering around the micelles limited the access of plasmin to casein.

Plasmin-induced hydrolysis has been reported to decrease or to be inhibited by the addition of denatured  $\beta$ -lactoglobulin but not by the addition of native  $\beta$ -lactoglobulin (Grufferty & Fox, 1986; Snoeren, van Riel, & Both, 1980). Sulphydryl–disulphide interchange reactions between the disulphide bridges of plasminogen and cysteine and/or  $\beta$ -lactoglobulin are known to decrease plasmin activity (Alichanidis et al., 1986; Grufferty & Fox, 1988c; Metwalli et al., 1998; Rollema & Poll, 1986; Ryan et al., 2012; Saint Denis et al., 2001) upon heat treatment. In the

present study, as plasmin was added after the high heat treatment, the chances of plasmin inhibition through sulphhydryl–disulphide interchange with whey proteins were decreased.

In the present study, plasmin was added externally to the heat-treated system, in which the whey proteins were attached to the casein micelles but plasmin was not; therefore, physical inhibition through the association of the whey proteins with the casein micelles (Enright & Kelly, 1999) played a major role in the decrease in plasmin activity. However, in real systems, plasmin and plasminogen activator are known to be attached to the casein micelle (de Rham & Andrews, 1982; Korycha-Dahl et al., 1983; Reimerdes & Klostermeyer, 1974; Richardson, 1983a); in this case, we believe that physical inhibition due to  $\beta$ -lactoglobulin would play a less significant role other than decreased activity through sulphhydryl–disulphide interchange reactions, as described earlier. The dissociation of proteins from the casein micelles upon heat treatment (Figure 55) was too small to show any effect on the hydrolysis rate; however, the dissociation of  $\kappa$ -casein might decrease the amount of whey proteins associated with the casein micelles and therefore could affect the plasmin-induced hydrolysis by decreasing the protective effect of the whey proteins. The heat treatment used for the study was much closer to the industrial retort sterilisation; however, by varying heating time the whey protein association and lactosylation much closer to the industrial UHT treatment were achieved.

## 7.5 CONCLUSIONS

Plasmin-induced hydrolysis was negatively affected by an increase in the heat treatment. Both the association of whey proteins with the casein micelles and lactosylation decreased the availability of protein to plasmin. These effects were compared and mechanisms were proposed. SPFM was the most resistant to plasmin, followed by SM and LFM, suggesting that lactosylation plays a more significant role than the association of whey proteins with the casein micelles in making protein resistant to plasmin-induced hydrolysis.

The present study can be useful in providing a method to control the plasmin activity in milk systems that are to be high heat treated. As the level of lactosylation and the association of whey proteins with the casein micelles can be manipulated/tailored by tuning the processing parameters, plasmin-induced hydrolysis can be controlled in real milk systems.



# Chapter 8: Plasmin-induced dissociation of the casein micelle

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## 8.1 INTRODUCTION

Sterilisation and ultra-high-temperature (UHT) treatment are commonly used in the dairy and food industries to increase the shelf life of milk and dairy beverages. However, heat-resistant enzymes such as plasmin and some bacterial proteases can partially survive UHT treatment, which may lead to defects in the texture and the taste of these products. Plasmin is a very heat-stable protease that is optimally active at about pH 7.5 and 37°C (Bastian & Brown, 1996; Grufferty & Fox, 1988b). Its activity and characteristics are similar to those of trypsin (plasmin and trypsin belong to the same alkaline serine protease family), with plasmin having greater specificity (Eigel et al., 1979; Fox, 1981b; Korycha-Dahl et al., 1983; Reimerdes, 1981; Richardson & Pearce, 1981). Both plasmin and trypsin hydrolyse proteins on the carboxyl site of Lys–X and Arg–X bonds, with a preference for Lys–X bonds (Kitchen, 1985).

Plasmin-induced proteolysis in milk products depends on many factors such as processing conditions, storage conditions, level of plasmin(ogen) and its activators, concentration of milk and other ingredients used in the product (Nielsen, 2003). UHT-treated milk systems with a pH range of 6.5–8 provide a good environment for plasmin activity; storage of such systems without refrigeration accelerates proteolysis and consequently results in bitterness and gelation because of residual plasmin activity (Chavan et al., 2011; Enright & Kelly, 1999; Kohlmann et al., 1988, 1991). Several authors (Kohlmann et al., 1991; Rauh et al., 2014a, 2014b; Venkatachalam et al., 1993) have commented on a possible role of plasmin in the age gelation of UHT milk. However, there appear to be limited studies on the role of plasmin separately from the storage-induced physicochemical changes in milk systems; furthermore, plasmin-induced dissociation of the casein micelles with respect to the release of different peptides from the micelle has not been well

studied. Therefore, the aim of the present work was to understand the plasmin-induced dissociation of casein micelles and the resulting physicochemical changes in milk systems, as opposed to storage-induced physicochemical changes, to provide greater understanding of the role of plasmin in sedimentation and gelation.

## **8.2 MATERIALS**

Skim milk (pH 6.65) was supplied by Fonterra Co-operative Group Ltd, Palmerston North, New Zealand. Plasmin from bovine plasma (5 U/mL) was purchased from Roche/Boehringer Mannheim GmbH (Germany). Chemicals for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), reverse-phase high performance liquid chromatography (RP-HPLC) and reverse-phase liquid chromatography–mass spectrometry (RP-LC–MS) buffers were purchased from Merck (Darmstadt, Germany). An acrylamide/bisacrylamide mixture 37.5:1 (2.6% C) from Bio-Rad (Hercules, CA, USA) was used. Soy trypsin inhibitor was purchased from Sigma–Aldrich (Auckland, New Zealand).

## **8.3 METHODS**

All experiments reported were repeated at least twice with the same milk samples. In addition, the experiments were repeated with several different milk samples. Although there were some variations between individual milks, the same trends and relationships as reported here were found for all samples examined to date.

### **8.3.1 Heat treatment**

The milk samples were equilibrated at 30°C before heat treatment. Subsamples (5 mL) of skim milk were transferred to glass vials and were heated at 120°C for 2 min {chosen to have minimum side reactions, i.e. lactosylation (<3%), but to have whey protein association (>50%) with the casein micelle, based on findings from Chapter 7}. The heat treatment was carried out in a thermostatically controlled oil bath fitted with a continuous rocking assembly. After heat treatment, the samples were cooled to room temperature by placing the vials in cold running water.

### **8.3.2 Plasmin-induced hydrolysis**

The milk samples were hydrolysed by adding plasmin at 15  $\mu\text{L/mL}$  (0.1 U/mL) of milk. The samples were incubated in a water bath at 37°C and were allowed to equilibrate to temperature for 1 h before plasmin addition; the pattern of hydrolysis was observed at incubation times of 0 min, 15 min, 30 min, 1 h, 2 h and 4

h. Sedimentation and/or gel formation were observed visually throughout the hydrolysis. The samples were removed from the water bath after each incubation time, sedimentation and/or gel formation were observed visually and the reaction was stopped immediately by adding trypsin inhibitor (2 mg/mL in water).

### **8.3.3 Particle size measurement**

Particle size measurements were carried out on all milk samples to observe the effect of heat treatment on the casein micelle size distribution in heated and non-heated milk systems. The particle size was measured by photon correlation spectroscopy using a Malvern Zetasizer 4 instrument and the associated ZET5110 particle sizing cell (Malvern Instruments Ltd, Malvern, Worcs., UK), by the method of Anema and Li (2003a). Milk samples (50  $\mu$ L) were dispersed in 5 mL of simulated milk ultrafiltrate before particle size measurement.

### **8.3.4 Turbidity measurement**

Turbidity measurements were performed using a Jasco V580 spectrophotometer (Japan Spectroscopic Co., Hachioji City, Japan) and techniques described previously (Chapter 6). The measurements were carried out in triplicate and average values were used in the analysis.

### **8.3.5 Centrifugation**

Centrifugation was carried out, using the method described previously (Chapter 7), to separate casein micelles and associated whey proteins from the serum phase. Subsequent protein analysis of these phases enabled the level of different caseins and peptides that had dissociated from the micelle into the serum phase to be identified.

### **8.3.6 SDS-PAGE**

To study the effect of plasmin-induced hydrolysis on the dissociation of the casein micelles and the generation of  $\gamma$ -casein [hydrophobic portion of  $\beta$ -casein –  $\beta$ -casein (f106/108–209)], SDS-PAGE was performed under reducing conditions, as described

previously (Bhatt et al., 2014), for both the whole sample and the serum phase of the sample. Dissociation from the casein micelle was presented as a percentage present in the serum phase of the total casein.

### **8.3.7 RP-HPLC**

The pattern of plasmin-induced hydrolysis was analysed by quantifying proteose peptones [hydrophilic portion of  $\beta$ -casein –  $\beta$ -casein(N-105/107)], as described previously (Bhatt et al., 2014), in both the whole (non-centrifuged) sample and the serum phase of the sample.

### **8.3.8 Mineral analysis**

The total calcium and phosphorus levels in both the serum phase and the milk were measured using the method of Hekmat and McMahan (1998). Milk and serum samples were digested with concentrated (16 mol/L) nitric acid by heating below their boiling points. Hydrogen peroxide (300 g/L) was added in droplets at the end of the digestion until a white ash was formed. This ash was then dissolved with 0.5 mL of 6 mol/L hydrochloric acid and diluted tenfold with distilled deionised water. The measurements were performed using simultaneous inductively coupled plasma–optical emission spectrometry (ICP–OES; Varian Vista-Pro, Mulgrave, Victoria, Australia) coupled to a V-groove nebuliser and equipped with a charge coupled device.

### **8.3.9 Mass spectrometry**

#### ***Sample preparation***

Mass spectrometry was used to identify the peptides in the serum phase at levels of hydrolysis of 5, 25 and 100%. Serum phase samples were collected as described in the centrifugation step. Each serum phase sample (500  $\mu$ L) was purified by solid phase extraction on a C18 cartridge (Sep-Pak, Waters). The eluate was concentrated by speed-vac centrifugation and was reconstituted in 50  $\mu$ L of 5% (v/v) acetonitrile, 0.2% (v/v) formic acid in water.

### ***LC-MS/MS of peptides***

Samples (5  $\mu$ L) were injected on to an UltiMate 3000 nano-flow UHPLC-System (Dionex, Thermo Scientific, CA, USA) that was in-line coupled to the nanospray source of an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, San Jose, CA, USA). Peptides were separated on an in-house-packed emitter-tip column [75  $\mu$ m internal diameter PicoTip fused silica tubing of length 8–9 cm (New Objective, Woburn, MA, USA) that was packed with C18 material (3  $\mu$ m beads, 100 Å pore size)] at a flow rate of 400 nL/min. The following gradient was used: (a) 5% solvent B [0.2% (v/v) formic acid in acetonitrile] in 95% solvent A [0.2% (v/v) formic acid in water] to 25% solvent B over 67 min; (b) 25% solvent B to 45% solvent B over 15 min; (c) 45% solvent B to 99% solvent B over 10 min.

#### *Typical instrument setting for the LTQ-Orbitrap*

Full MS in a mass range between  $m/z$  400 and  $m/z$  2000 was performed in the Orbitrap mass analyser with a resolution of 60,000 at  $m/z$  400 and an AGC (automated gain control) target of  $5e5$ . The preview mode for the FTMS master scan was enabled to generate precursor mass lists. The strongest six signals were selected for collision-induced dissociation-MS/MS in the LTQ ion trap at a normalised collision energy of 35% using an AGC target of  $2e4$  and one microscan. Dynamic exclusion was enabled with two repeat counts during 30 s and an exclusion period of 180 s. The exclusion mass width was set to 0.01.

#### ***Data analysis***

The raw data were processed through Proteome Discoverer software (version 1.4, Thermo Scientific) to generate peak lists in mgf (Mascot generic file) format using default settings, with the exception of the maximum precursor mass of 10,000. For protein identification, mgf files were searched against the bovine subset of the NCBI non-redundant amino acid sequence database (54,605 entries) using the Mascot search engine (<http://www.matrixscience.com>) with the following search settings: no enzyme was selected to allow for all possible cleavage sites; decoy search; oxidised methionine and phosphorylation of serine were selected as variable modifications. The precursor mass tolerance threshold was 10 ppm and the

maximum fragment mass error was 0.8 Da. The potential peptides generated by plasmin were recorded for the present study.

## 8.4 RESULTS AND DISCUSSION

### 8.4.1 Hydrolysis trend

Heat-treated and non-heat-treated milks were incubated with plasmin for 24 h. The level of hydrolysis increased with time and the hydrolysis patterns for  $\beta$ -casein and  $\alpha_s$ -casein ( $\alpha_{s1}$ -casein +  $\alpha_{s2}$ -casein) followed similar trends, in agreement with previous studies (Andrews, 1983; Srinivasan & Lucey, 2002), with  $\beta$ -casein hydrolysing more rapidly than  $\alpha_s$ -casein. In contrast, there was limited hydrolysis of  $\kappa$ -casein by plasmin, which is also in agreement with published observations (Eigel, 1977a; Srinivasan & Lucey, 2002). The resistance to hydrolysis could have been due to the presence of carbohydrate moieties attached to  $\kappa$ -casein (Doi et al., 1979) or to the effect of the amino acid sequence on the specificity of plasmin. In the present study, the hydrolysis of  $\beta$ -casein was analysed thoroughly in preference to the hydrolyses of  $\alpha_s$ -casein and  $\kappa$ -casein. Although both  $\beta$ -casein and  $\alpha_s$ -casein have high compositions in the casein micelle,  $\beta$ -casein was preferred because the peptides generated from  $\beta$ -casein are larger and well characterised, facilitating easier and thorough analysis. Both  $\beta$ -casein and  $\kappa$ -casein have distinct hydrophilic or hydrophobic regions; however,  $\beta$ -casein is the main substrate for plasmin activity whereas  $\kappa$ -casein is resistant (Bastian & Brown, 1996). The impacts of the level of  $\beta$ -casein hydrolysis on turbidity, particle size and protein dissociation were analysed.

$\beta$ -Casein has three main plasmin-sensitive bonds: Lys28–Lys29, Lys105–His106 and Lys107–Glu108. The hydrolysis by plasmin at these bonds results in the generation of the C-terminal peptides  $\gamma_1$ -casein (f29–209),  $\gamma_2$ -casein (f106–209) and  $\gamma_3$ -casein (108–209), with the corresponding N-terminal peptides (proteose peptones) PP8fast (f1–28), PP8slow (f29–105/107) and PP5 (f1–105/107) (Bastian & Brown, 1996).

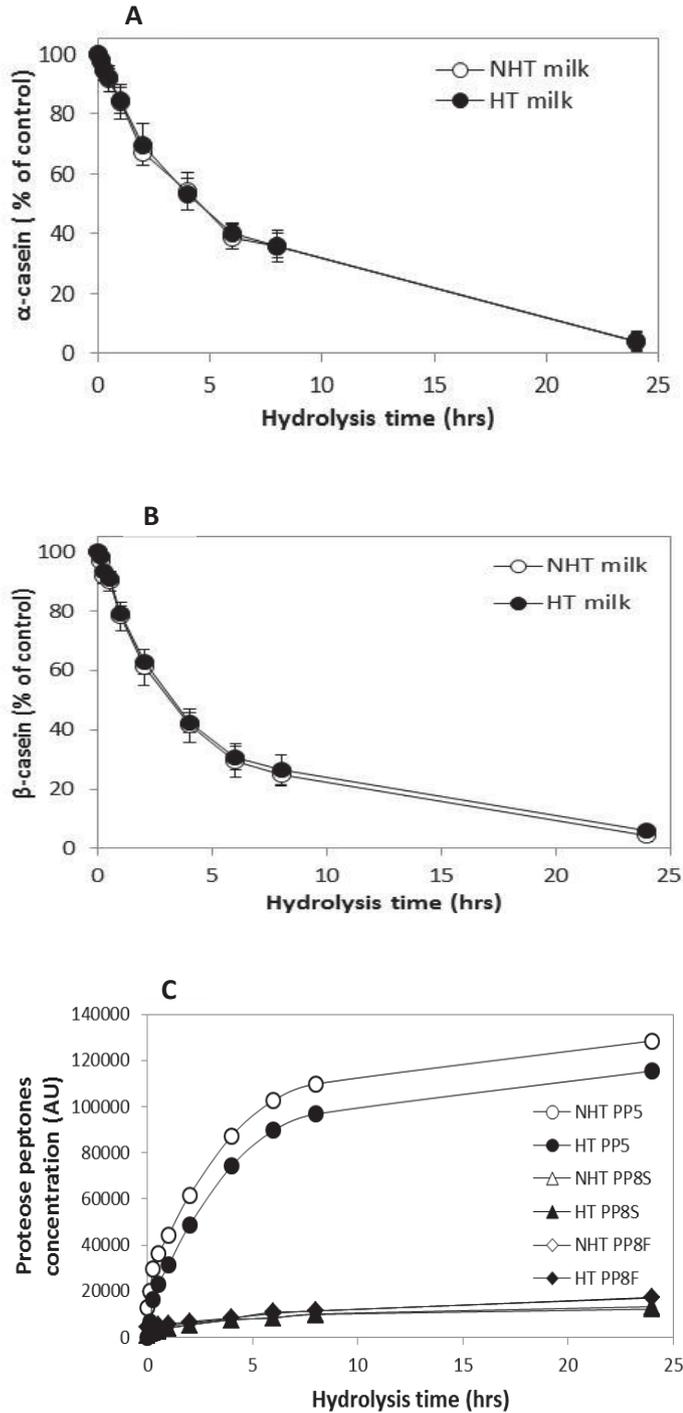


Figure 59. Plasmin-induced hydrolysis, as measured using SDS-PAGE, of: A,  $\alpha_s$ -casein ( $\alpha_{s1} + \alpha_{s2}$ -casein); B,  $\beta$ -casein. C, Generation of protease peptones during 24 h of hydrolysis as measured using RP-HPLC. NHT milk, non-heat-treated milk; HT milk, milk heat treated at 120°C for 2 min. Error bars refer to standard deviations.

The hydrolysis was slightly faster in the non-heat-treated samples than in the heat-treated samples in the initial stages of hydrolysis, as observed by the much faster

generation of PP5 in the non-heat-treated samples, which could have been due to decreases in the accessibility of the caseins to plasmin's action through the association of whey proteins with the casein micelle (Benfeldt et al., 1997; Bhatt et al., 2014; Enright & Kelly, 1999) and limited lactosylation, as observed in previous studies (Bhatt et al., 2014). Although trypsin-induced hydrolysis increased upon heat treatment (Leaver & Thomson, 1993), this could have been due to the relatively low molecular weight of trypsin (molecular weight = 24,000 Da, similar to that of casein, although trypsin has a globular tertiary structure, unlike casein) (Diaz, Gouldsworthy, & Leaver, 1996), which would not be expected to be affected by the hindrance caused by  $\beta$ -lactoglobulin- $\kappa$ -casein complexes on the casein micelle surface.

#### **8.4.2 Turbidity**

The plasmin-induced hydrolysis of milk leads to a change in the turbidity of the milk system. With an increasing level of hydrolysis, the turbidity of the milk samples decreased continuously in both the heat-treated and the non-heat-treated samples up to around 80%  $\beta$ -casein hydrolysis. The decrease in turbidity of the system can be explained by dissociation of the casein micelles upon plasmin-induced hydrolysis. The turbidity of the system depends on the size and the scattering factor of particles (Anema & Klostermeyer, 1997), and disintegration of the casein micelles as a result of plasmin-induced hydrolysis would have a negative impact on both the size and the scattering properties of the casein micelle. The scattering properties of the micelle could decrease because of decreases in density, as the micelle becomes increasingly hollow as a result of disintegration through plasmin-induced hydrolysis, ultimately resulting in a decrease in turbidity (Figure 60). These results are in agreement with observations based on  $L^*$ -values (Crudden, Afoufa-Bastien, Fox, Brisson, & Kelly, 2005a); Crudden et al. (2005a) found that plasmin-induced hydrolysis led to a decrease in the  $L^*$ -value (i.e. reduced opacity) of skim milk. The disintegration or dissociation of the casein micelle with increased pH (Anema & Creamer, 1993; Rajput, Bhavadasan, & Ganguli, 1983) and disruption of the colloidal calcium phosphate (CCP) (Mizuno & Lucey, 2005; Odagiri & Nickerson,

1964; Pitkowski et al., 2008) have already been reported to cause a decrease in turbidity.

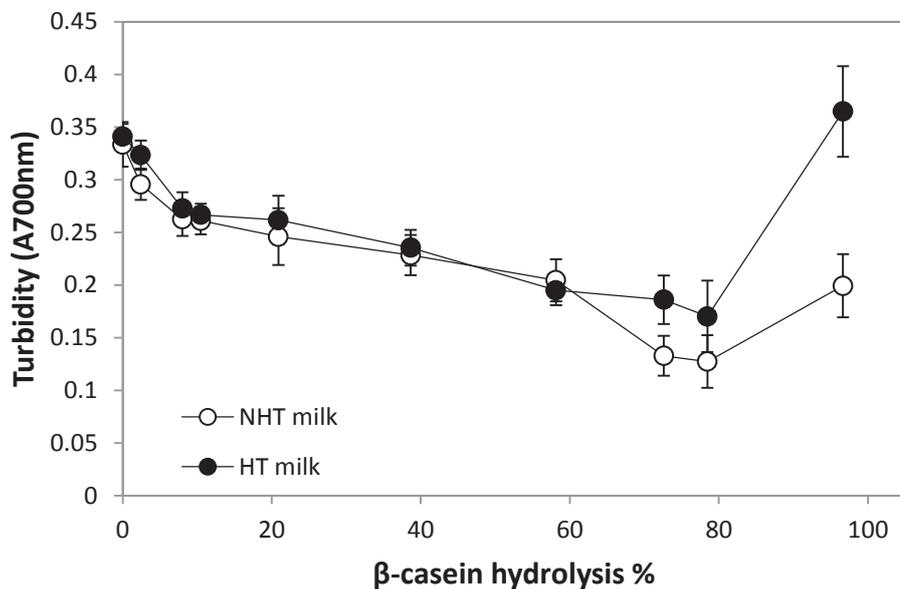


Figure 60. Effect of plasmin-induced hydrolysis on turbidity, as measured by absorbance at 700 nm in non-heat-treated (NHT) and heat-treated (HT) milks. Error bars refer to standard deviations.

Beyond 80% hydrolysis, the turbidity increased markedly. This increase could have been due mainly to the aggregation of proteins and hydrolysed peptides through hydrophobic interactions, leading to an increase in the average particle size (Figure 61). The increase in turbidity with heat treatment (Figure 60) is in agreement with previous observations (Anema & Klostermeyer, 1997; Jeurnink, 1992) and may be attributed primarily to the association of denatured whey proteins with  $\kappa$ -casein at the micelle surface (Jeurnink, 1992). The turbidity of the heat-treated samples was higher at the end of the hydrolysis, when they almost twice as turbid as the non-heat-treated samples. Such differences could have been due mainly to association of the thermally induced  $\beta$ -lactoglobulin- $\kappa$ -casein complexes with the plasmin-generated  $\gamma$ -caseins, resulting in much bigger complexes than in non-heated milk, in which  $\beta$ -lactoglobulin- $\kappa$ -casein complexes are absent.

### 8.4.3 Particle size and mean count rate

The particle size of both milk samples was found to decrease (Figure 61A) with an increase in the level of  $\beta$ -casein hydrolysis up to 80%. Interestingly, the trend was very similar to the turbidity trend and the trends correlated well. The underlying mechanism for both trends is likely to be attributable to plasmin-induced dissociation of the casein micelle. The casein micelle begins to disintegrate because of the hydrolysis, by plasmin, of  $\beta$ -casein and  $\alpha_s$ -casein, which are the major building blocks of the casein micelle.

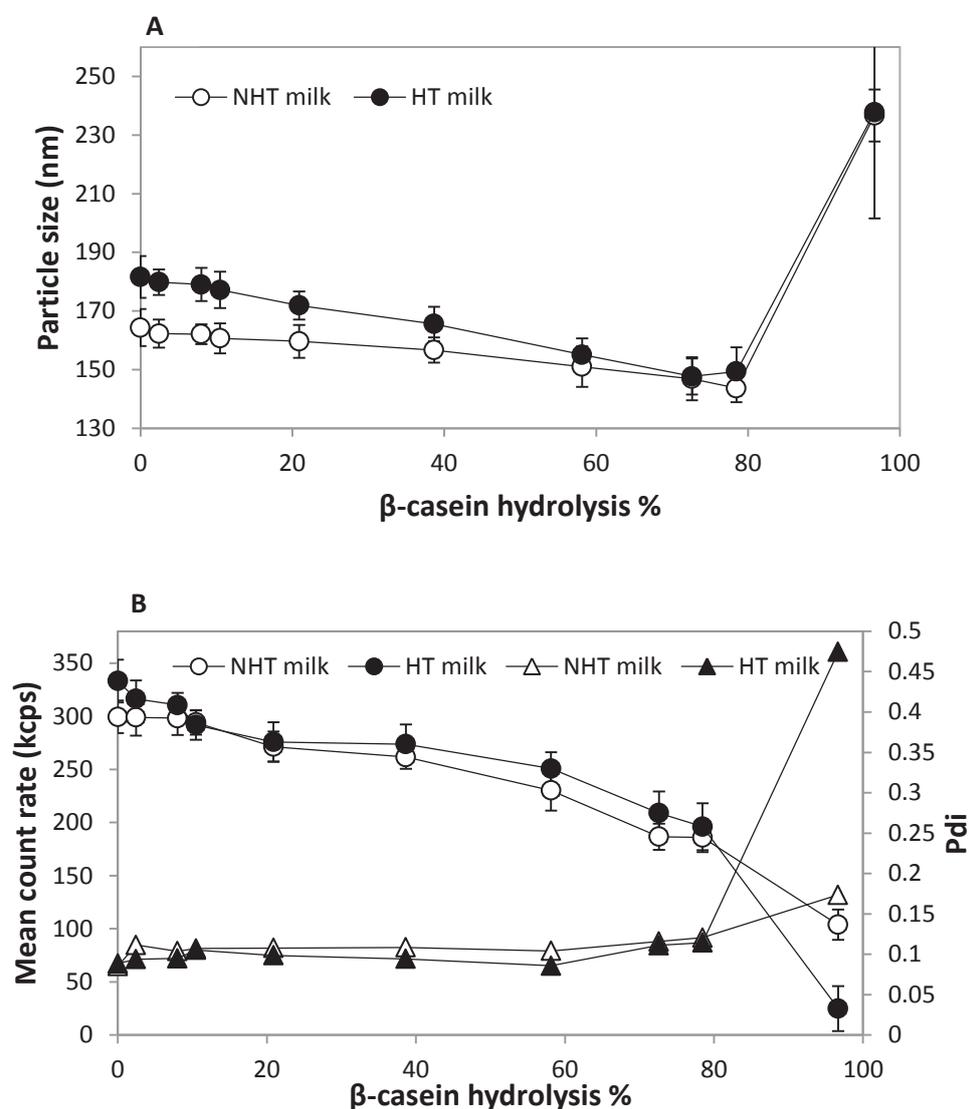


Figure 61. Effect of plasmin-induced hydrolysis on (A) particle size and (B) mean count rate and polydispersity index (PDI) of non-heat-treated (NHT) and heat-treated (HT) milks. Error bars refer to standard deviations.

In line with previous studies (Anema & Li, 2003a; Chapter 7), the heat-treated milk samples had a larger particle size than the non-heat-treated milk samples, which was thought to be due mainly to the heat-induced association of denatured whey proteins with the casein micelle and the formation of  $\beta$ -lactoglobulin- $\kappa$ -casein complexes (Creamer et al., 1978; Guyomarc'h et al., 2003; Jang & Swaisgood, 1990; Mohammad & Fox, 1987; Singh & Fox, 1987a).

Although the decrease in the particle size upon hydrolysis was faster in the heat-treated milk samples than in the non-heat-treated milk samples, the particle size of both systems became similar at a level of  $\beta$ -casein hydrolysis of around 80% (Figure 61A). With a further increase in hydrolysis, the particle size increased dramatically in both samples to a similar extent; however, the data for the heat-treated samples had much greater variation, as reflected in the standard deviation, which could have been due to the increase in the polydispersity index of the heat-treated samples (Figure 61B). The decrease in particle size at low levels of hydrolysis (up to 80% hydrolysis) and the increase in particle size at higher levels of hydrolysis are in agreement with Crudden et al. (2005a), who attributed the behaviour to the net change in the charge as a result of the hydrolysis of casein (i.e. hydrolysis results in a change in the isoelectric point of the remaining protein chain). Crudden et al. (2005a) proposed two possible hypotheses: the reduced charge on the casein micelles may have promoted the formation of aggregates, resulting in an increased apparent micelle size (and would have reduced the total number of particles); alternatively, as the micelles in the plasmin-treated milk were composed mostly of  $\gamma$ -caseins, proteose peptones, residual  $\alpha_{s1}$ -casein and  $\kappa$ -casein, their structure was presumably changed, e.g. becoming more loosely bound and therefore larger in size. Here, it is important to note that the increase in particle size was observed beyond 80%  $\beta$ -casein hydrolysis; in such extensively hydrolysed milk systems, the casein micelles will probably be completely disintegrated and, as a result, the measurement of the zeta potential will not be accurate. In contrast, the alternative hypothesis appears to be feasible and we believe that the hydrolysed hydrophobic peptides are likely to aggregate and lead to an observed increase in particle size.

The mean count rate decreased continuously, again suggesting dissociation of the casein micelle, whereas there was not much change in the polydispersity index up to 80%  $\beta$ -casein hydrolysis for both the heat-treated and the non-heat-treated milk samples (Figure 61B). Beyond 80% hydrolysis, the polydispersity index of the heat-treated samples increased dramatically compared with that of the non-heat-treated samples, which could have been due to release and aggregation of  $\beta$ -lactoglobulin- $\kappa$ -casein complexes through hydrophobic interactions in the heat-treated milk samples.

#### **8.4.4 Release of smaller peptides from the casein micelle**

The release or dissociation of peptides from the casein micelle as a result of plasmin-induced hydrolysis was analysed using LC-MS/MS. At the end of the plasmin-induced hydrolysis of milk, the numbers of peptides identified in the serum phase were from:  $\alpha_{s2}$ -casein, 20 peptides;  $\alpha_{s1}$ -casein, 19 peptides;  $\beta$ -casein; 15 peptides;  $\beta$ -lactoglobulin, 7 peptides;  $\kappa$ -casein, 5 peptides.

The  $\alpha_{s1}$ -casein family constitutes up to 40% of the casein fraction in bovine milk.  $\alpha_{s1}$ -Casein is a single-chain protein with no cysteinyl (Cys) residues. It consists of 199 amino acid residues with a calculated molecular weight of 23,615 Da (Swaigood, 2003). Peptides generated from hydrolysis at Lys4-His5, Lys8-His9, Arg22-Phe23, Lys34-Glu35, Lys36-Val37, Lys42-Asp43, Lys79-His80, Arg90-Tyr91, Lys102-Lys103, Lys103-Tyr104, Lys105-Val106 and Arg119-Leu120 were rapidly released from the micelle at the 5% hydrolysis level (Figure 62, Table 13). Sites Arg22-Phe23, Arg90-Tyr91, Lys102-Lys103, Lys103-Tyr104, Lys105-Val106 and Arg119-Leu120 have previously been identified as principal cleavage sites for plasmin (McSweeney et al., 1993), which is consistent with the rapid release of peptides from hydrolysis at these sites in our study. According to McSweeney et al. (1993), the initial cleavage of  $\alpha_{s1}$ -casein occurs at the centre of the molecule but we also observed the release of peptides from the N-terminal region of  $\alpha_{s1}$ -casein, in agreement with observations on the limited trypsinolysis of the casein micelle by Diaz et al. (1996). These differences could have been due to the use of pure  $\alpha_{s1}$ -

casein and hydrolysis at pH 8.4 by McSweeney et al. (1993); in our study, the  $\alpha_{s1}$ -casein was present in casein micelles and was hydrolysed at pH 6.6.

In contrast, peptides generated from hydrolysis at Lys84–Glu85, Arg100–Leu101, Lys124–Glu125, Lys132–Glu133 and Arg151–Gln152 were released slowly from the micelle and were present only in fully hydrolysed samples (Table 13), suggesting much slower hydrolysis and release from the casein micelle. Hydrolysis at Arg151–Gln152 was in agreement with McSweeney et al. (1993) but in contrast to Rauh et al. (2014b), which could have been due to precipitation of the generated peptides at pH 4.6 (McSweeney et al., 1993). Although Lys58–Gln59 and Lys79–His80 were hydrolysed and were present in a very polar domain (Ser41–His80) with an approximate net charge of  $-20.6$  at pH 6.6 (Swaisgood, 2003),  $\alpha_{s1}$ -casein(f59–79) was absent at all levels of hydrolysis, in agreement with previous studies (McSweeney et al., 1993; Rauh et al., 2014b). This absence of  $\alpha_{s1}$ -casein(f59–79) could have been due mainly to the presence of four phosphoserine residues and their strong calcium binding (Cross, Huq, Palamara, Perich, & Reynolds, 2005) and involvement in the formation of CCP, preventing it from being released from the casein micelle. Peptides from the region Gln152–Trp199 were not present at any level of hydrolysis. This could have been due to the presence of only one plasmin-sensitive residue, Lys193, in this region. Furthermore, the hydrophobic region around Pro168, including Trp164, is involved in higher order associations (Swaisgood, 2003) in the casein micelle and could limit the accessibility of plasmin, suggesting that peptides from this region could remain associated with the rest of the casein micelle and not be dissociated from it. These observations are in agreement with Gagnaire and Léonil (1998) on trypsinolysis, as cleavage of Lys193–Thr194 has been observed previously (Gagnaire & Léonil, 1998; Le Bars & Gripon, 1993; McSweeney et al., 1993; Rauh et al., 2014b).

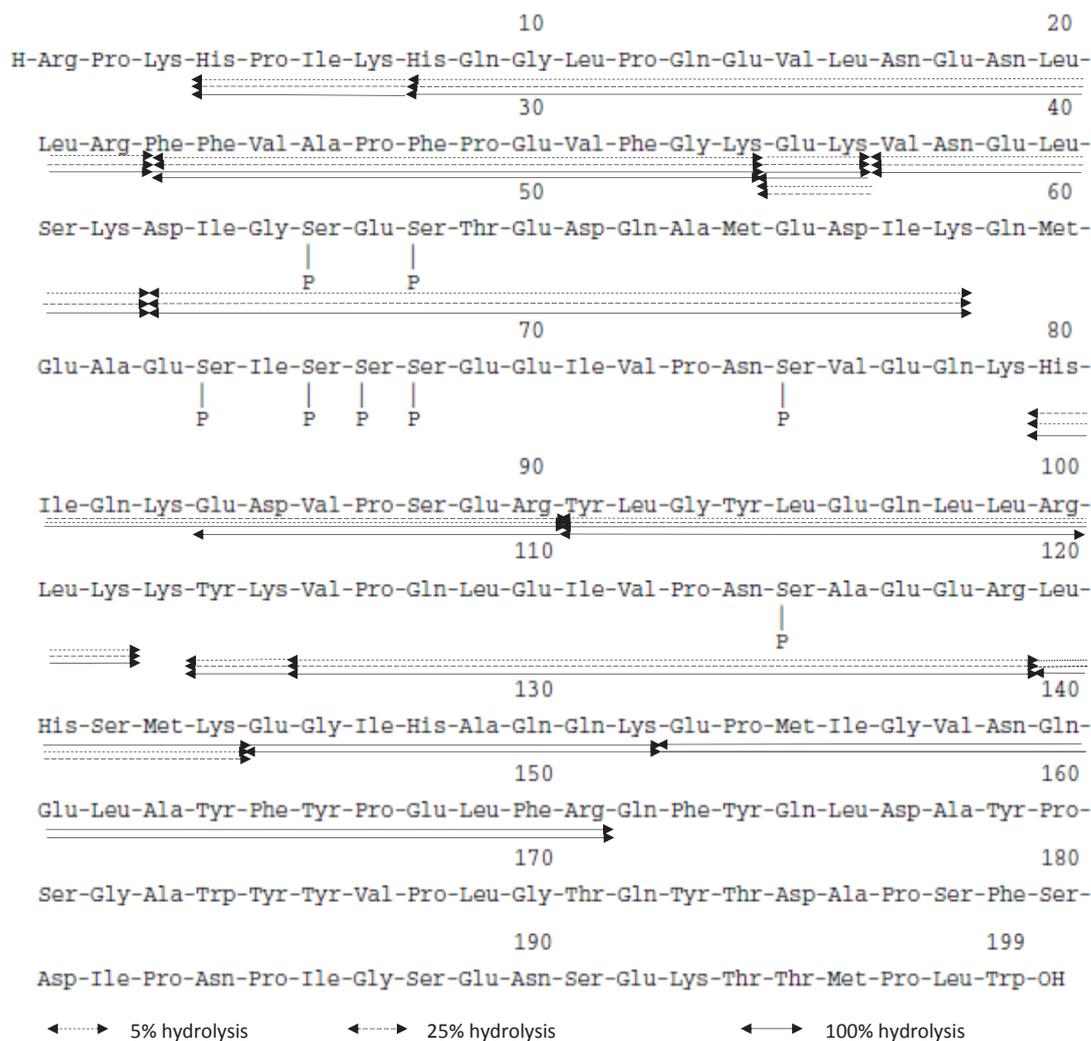


Figure 62. Schematic representation of the primary structure of  $\alpha_{s1}$ -casein B 8P and the peptides released from the casein micelle upon 5, 25 and 100% plasmin-induced hydrolysis in non-heat-treated milk at 37°C. The peptides generated are represented by arrows.

Table 13. Identification of  $\alpha_{s1}$ -casein peptides released from the casein micelle upon 5, 25 and 100% plasmin-induced hydrolysis in non-heat-treated milk

Sequence	Observed mass	Calculated mass	Ion score	Modification	Sequence	Presence at hydrolysis level		
						5%	25%	100%
4-22	2234.24	2234.23	37		K.HPIKHQGLPQEVNENLLR.F	X	X	X
8-22	1758.95	1758.94	52		K.HQGLPQEVNENLLR.F	X	X	X
8-34	3124.66	3124.65	41		K.HQGLPQEVNENLLRFFVAPFVFGK.E			X
23-34	1383.72	1383.72	52		R.FFVAPFVFGK.E	X	X	X
23-36	1640.86	1640.86	32		R.FFVAPFVFGKEK.V	X	X	X
35-42	945.51	945.51	30		K.EKVNELSK.D	X	X	X
	1025.48	1025.48	26	P	K.EKVNELSK.D			X
37-58	2437.14	2437.12	71	P	K.VNELSKDIGSESTEDQAMEDIK.Q	X	X	X
	2597.07	2597.05	64	2P	K.VNELSKDIGSESTEDQAMEDIK.Q			X
43-58	1846.73	1846.72	63	P	K.DIGSESTEDQAMEDIK.Q	X	X	X
80-90	1336.67	1336.67	42		K.HIQKEDVPSEY	X	X	X
84-100	2079.08	2079.06	35		K.EDVPSERYLGYLEQLLR.L			X
91-100	1266.70	1266.70	61		R.YLGYLEQLLR.L			X
91-102	1507.88	1507.88	44		R.YLGYLEQLLRK.K	X	X	X
104-119	1950.96	1950.95	79	P	K.YKVPQLEIVPNSAEER.L	X	X	X
106-119	1579.83	1579.82	47		K.VPQLEIVPNSAEER.L	X	X	X
106-124	2272.11	2272.09	52	P	K.VPQLEIVPNSAEERLHSMK.E	X	X	X
120-132	1505.79	1505.78	61		R.LHSMKEGIHAQQK.E			X
125-132	909.47	909.47	39		K.EGIHAQQK.E			X
125-151	3206.61	3206.59	113		K.EGIHAQQKKEPMIGVNQELAYFYPELFR.Q	X		X
133-151	2315.13	2315.13	68		K.EPMIGVNQELAYFYPELFR.Q	X		X

$\beta$ -Casein (i.e. A2-5P) is a single polypeptide chain with no Cys residues and containing 209 residues with a calculated molecular weight of 23,983 Da (Farrell et al., 2004).  $\beta$ -Casein is the most hydrophobic of the caseins, having an N-terminal sequence of charged amino acids as well as a phosphoserine cluster and having the second half of the molecule neutral and containing hydrophobic amino acid residues (Farrell et al., 2004).

Fifteen  $\beta$ -casein peptides were released on complete plasmin-induced hydrolysis of the casein micelle (Table 14). All potential plasmin-sensitive sites in  $\beta$ -casein were hydrolysed with the exception of Arg1–Glu2 and Arg202–Gly203, in agreement with studies on trypsin (Diaz et al., 1996; Gagnaire & Léonil, 1998), suggesting high accessibility. Of the eight peptides from the hydrophilic region 1–107, six were present in the 5% hydrolysed sample, indicating rapid hydrolysis and/or the release of peptides from the hydrophilic region, in agreement with the observation of much faster release from the casein micelle of proteose peptones than of  $\gamma$ -casein. Seven peptides were released from the hydrophobic region 107–209 at the end of hydrolysis; only one peptide,  $\beta$ -casein(f184–209), was released from the C-terminal end at 5% hydrolysis and  $\beta$ -casein(f177–183) was released at 25% hydrolysis (Figure 63, Table 14). The much slower release of peptides from the hydrophobic region is in agreement with the much slower release of the hydrophobic  $\gamma$ -casein peptides from the micelle and less accessibility of the lysine residues in the hydrophobic regions.

Peptide  $\beta$ -casein(f1–28) was released rapidly and all four serine residues were phosphorylated, which could have been due to some of these fractions being very close to the surface of the casein micelle (Leaver & Thomson, 1993), or loosely bound to the micelle through weak CCP interactions (Gagnaire & Léonil, 1998) or pre-existing in the serum phase. Gagnaire and Léonil (1998) observed complete retention of  $\beta$ -casein(f1–105/107) (PP5) and  $\beta$ -casein(f184–209) in the casein micelle during trypsinolysis, whereas we observed rapid release of these peptides from the casein micelle even at 5% hydrolysis (Table 14).

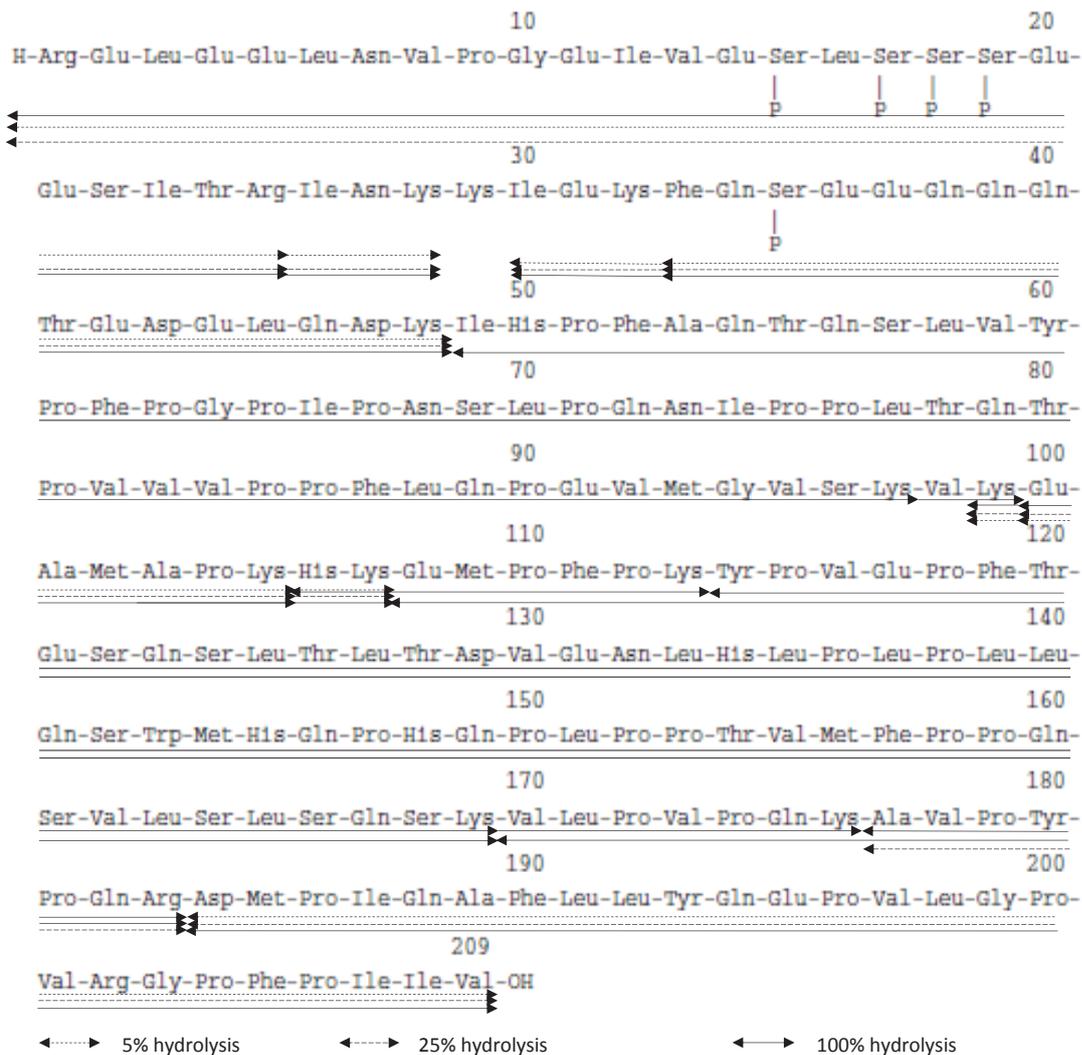


Figure 63. Schematic representation of the primary structure of  $\beta$ -casein A2 and the peptides released from the casein micelle upon 5, 25 and 100% plasmin-induced hydrolysis in non-heat-treated milk at 37°C. The peptides generated are represented by arrows.

Table 14. Identification of  $\beta$ -casein peptides released from the casein micelle upon 5, 25 and 100% plasmin-induced hydrolysis in non-heat-treated milk

Sequence	Observed mass	Calculated mass	Ion score	Modification	Sequence	Presence at hydrolysis level		
						5%	25%	100%
1–25	2801.42	2801.39	87		-RELEELNVPGEIVESLSSEESITR.I	X	X	X
1–28	3121.29	3121.26	50	4P	-RELEELNVPGEIVESLSSEESITR.I			X
	3236.61	3236.58	64	P	-RELEELNVPGEIVESLSSEESITRINK.K	X	X	X
	3316.57	3316.55	64	2P	-RELEELNVPGEIVESLSSEESITRINK.K	X	X	X
	3396.53	3396.51	80	3P	-RELEELNVPGEIVESLSSEESITRINK.K	X	X	X
	3476.48	3476.48	60	4P	-RELEELNVPGEIVESLSSEESITRINK.K	X	X	X
30–48	2431.05	2431.04	81	P	K.IEKFSQQQTEDELQDK.I	X	X	X
33–48	1980.86	1980.85	95	P	K.FQSEEQQTEDELQDK.I	X	X	X
49–97	5331.85	5331.84	37		K.IHPFAQTQSLVYPPFGPIPNLQNIPLTQTPVWVPPFLQPEVMGVSK.V			X
49–99	5543.04	5543.01	54		K.IHPFAQTQSLVYPPFGPIPNLQNIPLTQTPVWVPPFLQPEVMGVSKV.K.E			X
98–105	872.8	872.48	58		K.VKEAMAPK.H	X	X	X
100–107	910.48	910.47	30		K.EAMAPKH.E	X	X	X
106–113	1012.52	1012.52	35		K.HKEMPPFK.Y			X
108–169	7087.62	7087.6	32		K.EMFPKYPVEFTESQSLTLDVENLHPLPQLQSWMHQPHQLPPTVMFPFQSVLSQSK.V			X
114–169	6358.26	6358.25	20		K.YPVEFTESQSLTLDVENLHPLPQLQSWMHQPHQLPPTVMFPFQSVLSQSK.V			X
114–176	7119.74	7119.73	20		K.YPVQFTESQSLTLDVENLHPLPQLQSWMHQPHQLPPTVMFPFQSVLSQSKVLPVPEK.A			X
170–183	1590.93	1590.92	55		K.VLPVQKAVYPQR.D			X
177–183	829.45	829.44	17		K.AVYPQR.D		X	X
184–209	2908.6	2908.59	62		R.DMPIQAFLLYQEPVLGVPVRGPFPII.V.-	X	X	X

$\alpha_{S2}$ -Casein consists of 207 amino acids with a molecular weight of 25,226 Da for the A variant with 11 phosphorylated serine residues (Farrell, Malin, Brown, & Mora-Gutierrez, 2009). Twenty peptides were identified in the serum phase of the completely hydrolysed sample whereas only eight peptides were present in the serum phase of the 5% hydrolysed sample (Table 15, Figure 64). The cleavage sites were in agreement with previous studies (Le Bars & Gripon, 1989; Visser et al., 1989b). N-terminal peptide  $\alpha_{S2}$ -casein(f1–21), peptides from the middle,  $\alpha_{S2}$ -casein(f115–125),  $\alpha_{S2}$ -casein(f115–136),  $\alpha_{S2}$ -casein(f138–149),  $\alpha_{S2}$ -casein(f138–150) and  $\alpha_{S2}$ -casein(f174–181), and C-terminal peptides  $\alpha_{S2}$ -casein(f182–188) and  $\alpha_{S2}$ -casein(f198–205) were present in the 5% hydrolysed sample (Table 15), suggesting preferential hydrolysis at these bonds and rapid release of these peptides from the casein micelle, in agreement with the observation in previous studies that the C terminus, the N terminus and the middle of the molecule are relatively accessible to trypsin (Diaz et al., 1996; Gagnaire & Léonil, 1998). In contrast, only four of the 12 peptides from residues 150–207 were present at 5% hydrolysis, which could have been mainly because the high hydrophobicity of the chain in the 171–207 region (Farrell et al., 2009) made them associate with other hydrophobic chains in the casein micelle. Rauh et al. (2014b) observed the generation of peptides from residues 182–207 after only 4 weeks of storage, suggesting sensitivity of the C-terminal region to hydrolysis; however, we observed slower release of these peptides from the casein micelle, possibly because of the high hydrophobicity of this region (Farrell et al., 2004).

It was also observed that, of the three peptides generated from the 151–173 sequence, none was present in the 5 and 25% hydrolysed samples, which could have been due to the involvement of fragment 158–173 (six positive charges) in ionic interactions with other caseins (Tauzin, Miclo, Roth, Mollé, & Gaillard, 2003). The positive C-terminal tail of  $\alpha_{S2}$ -casein has already been suggested to have ionic interactions with  $\kappa$ -casein (Kudo & Mada, 1983). Of four peptides from the 25–113 sequence, none was present at 5% hydrolysis and two,  $\alpha_{S2}$ -casein(f71–80) and  $\alpha_{S2}$ -casein(f81–91), were present in the 25% hydrolysed sample, which could have been due to less sensitivity to plasmin (Le Bars &

Gripon, 1989) or because the hydrophobicity of the region 81–125 (Farrell et al., 2009) did not allow plasmin to access or release the hydrolysed peptides.  $\alpha_{s2}$ -Casein(f92–113) was present only in the fully hydrolysed sample, in agreement with Tauzin et al. (2003); this was attributed to its high hydrophobicity (37.1 kcal/mol) (Gourley et al., 1998). Peptides generated from bonds Lys1–Asn2 and Lys191–Pro192 were absent at all levels of hydrolysis, in agreement with the observations of Tauzin et al. (2003), suggesting resistance of these bonds to hydrolysis by plasmin and trypsin. Rauh et al. (2014b) observed the absence of peptides generated from cleavage sites Lys91–Phe92, Lys32–Glu33 and Lys41–Glu42 in milk after hydrolysis by plasmin. However, in our work, the presence of peptides generated from Lys91–Phe92 and Lys32–Glu33 suggests that these sites are accessible to plasmin, whereas Lys41–Glu42 cleavage was not observed, in agreement with Rauh et al. (2014a). No peptide was released from the 33–70 sequence, suggesting either no hydrolysis at the Lys41–Glu42 and Arg45–Asn46 bonds or that the peptides generated were not released from the micelle. However, although hydrolysis at the Lys32–Glu33 and Lys70–Ile71 bonds should generate (f33–70), it was absent, suggesting a higher probability of the peptides not being released from the micelle. This could have been due to the presence of three phosphoserine residues and their involvement in CCP formation and/or intramolecular disulphide bond formation because of the presence of Cys36 and Cys40 (Farrell et al., 2009; Rasmussen, Højrup, & Petersen, 1994). According to Rauh et al. (2014b), the cleavage of Arg45–Asn46, Lys70–Ile71, Lys80–Ile81, Arg125–Glu126, Lys166–Ile167 and Arg205–Tyr206 has not previously been reported for plasmin, either in model studies or as part of the indigenous peptide profile in milk; however, in our study, we observed cleavage at all these sites except Arg45–Asn46, which is in agreement with studies using trypsin by Tauzin et al. (2003). Interestingly, the release of peptide  $\alpha_{s2}$ -casein(f1–21) was observed to be affected by phosphorylation; peptides with two or more phosphorylated residues were absent at 5% hydrolysis and the peptide with four phosphorylated residues was present only in the fully hydrolysed sample (Table 15).



Figure 64. Schematic representation of the primary structure of  $\alpha_{s2}$ -casein and the peptides released from the casein micelle upon 5, 25 and 100% plasmin-induced hydrolysis in non-heat-treated milk at 37°C. The peptides generated are represented by arrows.

Table 15. Identification of  $\alpha_{s2}$ -casein peptides released from the casein micelle upon 5, 25 and 100% plasmin-induced hydrolysis in non-heat-treated milk

Sequence	Observed mass	Calculated mass	Ion score	Modification	Sequence	Presence at hydrolysis level		
						5%	25%	100%
1-21	2426.14	2426.13	102		-KNTMEHVSSEESIISQETK.Q	X	X	X
	2506.10	2506.09	64	P	-KNTMEHVSSEESIISQETK.Q	X	X	X
	2586.08	2586.06	69	2P	-KNTMEHVSSEESIISQETK.Q		X	X
	2666.04	2666.03	29	3P	-KNTMEHVSSEESIISQETK.Q		X	X
	2746.02	2745.99	25	4P	-KNTMEHVSSEESIISQETK.Q			X
25-32	873.44	873.44	20		K.NMAINPSK.E			X
71-80	1245.64	1245.64	53		K.ITVDDKHYQK.A		X	X
81-91	1366.70	1366.69	53		K.ALNEINQFYQK.F		X	X
92-113	2708.40	2708.40	15		K.FPQYLQLYQGPIVLPWDQVK.R			X
115-125	1194.68	1194.67	44		R.NAVPIPTLNR.E	X	X	X
115-136	2507.21	2507.19	94		R.NAVPIPTLNREQLSTSEENSK.K	X	X	X
	2507.21	2507.19	69	P	R.NAVPIPTLNREQLSTSEENSK.K			X
	2587.18	2587.16	31	2P	R.NAVPIPTLNREQLSTSEENSK.K			X
137-149	1593.70	1593.70	66	P	K.KTVDMESTEVEFTK.K			X
138-149	1385.65	1385.64	79		K.TVDMESTEVEFTK.K	X	X	X
	1465.61	1465.60	77	P	K.TVDMESTEVEFTK.K	X	X	X
138-150	1593.71	1593.70	42	P	K.TVDMESTEVEFTKTK.L	X	X	X
151-158	976.51	976.51	38		K.TKLTSEEK.N			X
167-173	921.51	921.50	35		K.ISQRYQK.F			X
171-181	1397.78	1397.77	48		R.YQKFALPQYLK.T			X
174-181	948.55	978.55	26		K.FALPQYLK.T	X	X	X
182-188	902.46	902.46	30		K.TVYQHQA	X	X	X
182-197	1982.04	1982.06	32		K.TVYQHQAAMKPWIQPK.T			X
189-197	1097.61	1097.61	38		K.AMKPWIQPK.T		X	X
189-199	1326.75	1326.75	27		K.AMKPWIQPKTK.V			X

198-205	974.60	974.59	43	K.TKVIPYVR.Y	X	X	X
200-207	1021.60	1021.60	18	K.VIPYRYL.-			X

$\kappa$ -Casein and whey proteins are known to be resistant to plasmin (Bastian & Brown, 1996) and none of the peptides appeared at 5% hydrolysis. Only two peptides from  $\kappa$ -casein (Table 16) and four peptides from  $\beta$ -lactoglobulin (Table 17) were released into the serum phase at 25% hydrolysis. At the end of the hydrolysis, five peptides from  $\kappa$ -casein and seven peptides from  $\beta$ -lactoglobulin were identified in the serum phase.

Table 16. Identification of  $\kappa$ -casein peptides released from the casein micelle upon 5, 25 and 100% plasmin-induced hydrolysis in non-heat-treated milk

Sequence	Observed mass	Calculated mass	Ion score	Modification	Sequence	Presence at hydrolysis level		
						5%	25%	100%
14–21	1042.48	1042.47	42		K.DERFFSDK.I	X	X	X
17–24	954.52	954.52	60		R.FFSDKIAK.Y	X	X	X
25–34	1250.70	1250.70	17		K.YPIQYVLSR.Y			X
25–68	5241.79	5241.75	33		K.YPIQYVLSRPSYGLNYYQQKPVALLNNQFLPYPYAKPAAVR.S			X
69–86	1979.10	1979.08	23		R.SPAQILQWQVLSNTVPAK.S			X

Table 17. Identification of  $\beta$ -lactoglobulin peptides released from the casein micelle upon 5, 25 and 100% plasmin-induced hydrolysis in non-heat-treated milk

Sequence	Observed mass	Calculated mass	Ion score	Modification	Sequence	Presence at hydrolysis level		
						5%	25%	100%
1–8	932.54	932.54	64		-LIVTQTMK.G	X	X	X
1–14	1586.92	1586.91	48		-LIVTQTMKGLDIQK.V	X	X	X
71–77	801.50	801.50	25		K.IIAEKT.K			X
84–91	915.47	915.47	39		K.IDALNENK.V	X	X	X
84–100	1962.05	1962.03	78		K.IDALNENKVLVLDTDYK.K			X
92–100	1962.05	1962.03	78		K.VLVLDTDYK.K	X	X	X
142–148	836.47	836.47	33		K.ALPMHIR.L			X

### 8.4.5 Release of $\gamma$ -casein and $\kappa$ -casein from the casein micelle

Plasmin-induced dissociation of  $\kappa$ -casein and the hydrolysed peptide  $\gamma$ -casein ( $\gamma_2$ -casein +  $\gamma_3$ -casein) from the casein micelle was measured using SDS-PAGE. With an increase in the hydrolysis time, the concentration of  $\gamma$ -casein increased because of the hydrolysis of  $\beta$ -casein. The dissociation of the hydrolysed peptide  $\gamma$ -casein –  $\beta$ -casein(f106/108–209) – increased gradually with an increase in hydrolysis (Figure 65A) and reached a maximum upon the complete hydrolysis of  $\beta$ -casein.

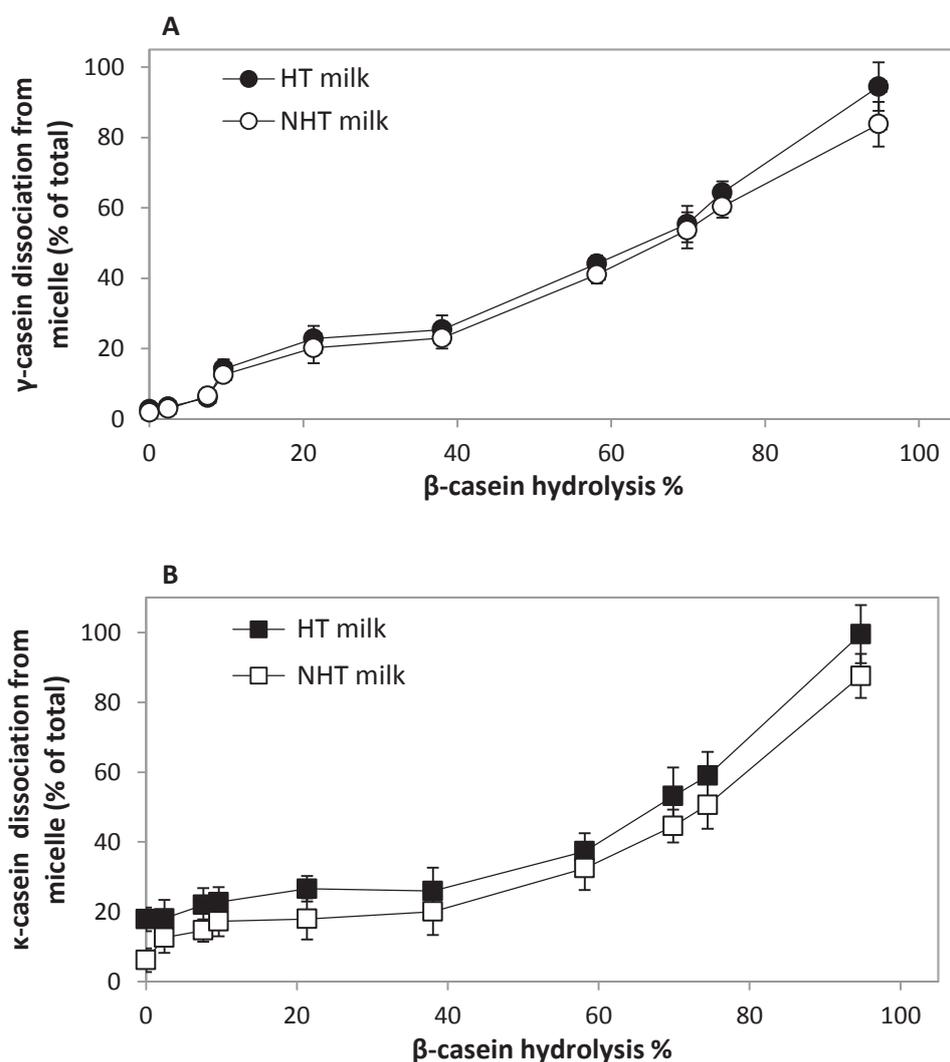


Figure 65. Effect of plasmin-induced hydrolysis on the dissociation patterns of (A)  $\gamma$ -casein and (B)  $\kappa$ -casein in non-heat-treated (NHT) and heat-treated (HT) milks, quantified using SDS-PAGE. Error bars refer to standard deviations.

This retention of the hydrophobic peptide  $\gamma$ -casein –  $\beta$ -casein(f106/108–209) – with the casein micelle is in agreement with previous studies on trypsinolysis (Gagnaire & Léonil, 1998; Yoshikawa, Sugimoto, & Chiba, 1975). According to the dual-binding model of micellar assembly and structure,  $\kappa$ -casein can link hydrophobically into growing chains through its hydrophobic N-terminal block, and the growth of polymer clusters is terminated by the C-terminal hydrophilic block because of the absence of phosphoserine clusters (Horne, 2008). The outer layer of the casein micelle is dominated by  $\kappa$ -casein although other caseins are also present at, or close to, the surface (Dalgleish, 1998); therefore, it can be said that  $\kappa$ -casein is anchored to the micelle through hydrophobic regions of  $\alpha_s$ - and  $\beta$ -caseins, i.e.  $\gamma$ -casein. The presence of  $\kappa$ -casein on the surface and the extension of its C-terminal hydrophilic chain forms a hairy layer on  $\kappa$ -casein, also known as a polyelectrolyte brush, which provides steric stabilisation to the casein micelle (de Kruif & Zhulina, 1996). We observed a pattern of  $\kappa$ -casein dissociation upon hydrolysis that was similar to that of  $\gamma$ -casein, and the dissociation increased slowly with an increase in the hydrolysis time (Figure 65B). It is known that  $\kappa$ -casein can form disulphide-linked oligomeric aggregates (Groves, Wickham, & Farrell, 1998); it could be present on the micellar surface in this form, so that the micellar surface might be covered by bunches of hairs, rather than single hairs, and it is likely that these polymers would have greater attachment/linkage to the rest of the micelle structure, making them more difficult to remove.

The level of dissociated  $\kappa$ -casein was higher in the heat-treated non-hydrolysed sample than in the non-heat-treated non-hydrolysed sample (Figure 65B), which could have been due mainly to heat-induced dissociation of  $\kappa$ -casein from the casein micelle, as proposed by several authors (Anema, 1998; Anema & Klostermeyer, 1997; Anema & Li, 2000; Singh & Fox, 1985). There was slightly greater dissociation of both  $\gamma$ -casein (Figure 65A) and  $\kappa$ -casein (Figure 65B) in the heat-treated sample than in the non-heat-treated sample throughout the hydrolysis. This greater dissociation in the heat-treated samples can be attributed mainly to the increased dissociation of  $\kappa$ -casein in the heat-treated samples throughout the hydrolysis and because, with this heat-induced dissociation, the

now dissociated  $\kappa$ -casein probably cannot keep the  $\gamma$ -casein attached to the micelle through hydrophobic interactions and/or through hindrance by the polymeric hairy layer. The identical trends for the dissociations of  $\kappa$ -casein and  $\gamma$ -casein indicated that these proteins might be dissociated together in an associative form because of their linkage through hydrophobic interactions. This theory is supported by an understanding of the mechanism of attachment. Holt (1992) proposed that a significant proportion of  $\beta$ -casein is bound to  $\kappa$ -casein and may be involved in the internal cohesion of the casein micelles. The dissociation of  $\gamma$ -casein from the micelle depends on the breakage of the anchoring point at Lys105 and/or Lys107 of  $\beta$ -casein. If this portion of  $\beta$ -casein is the dominant site of  $\kappa$ -casein attachment, as proposed by Holt (1992), then it is logical that  $\kappa$ -casein and  $\gamma$ -casein would dissociate in tandem.

#### **8.4.6 Release of proteose peptones from the casein micelle**

The generation of proteose peptone and its dissociation from the micelle were measured using RP-HPLC (Figure 66). With increasing hydrolysis, the dissociation of proteose peptone increased, in agreement with the dissociation of the casein micelle, as observed by turbidity and particle size measurements.

The percentage of dissociated proteose peptone was much higher than that of  $\gamma$ -casein from the start of the hydrolysis (Figure 66). This difference between the dissociation patterns of proteose peptones and  $\gamma$ -casein could be attributed mainly either to their hydrophobicity or to their accessibility. Proteose peptones are hydrophilic and, on hydrolysis, are readily released from the micelle through the porous micellar structure if they are not attached to CCP. In contrast, the presence of  $\kappa$ -casein in polymeric form on the micelle surface could inhibit the release of  $\gamma$ -casein, either through hydrophobic interactions or through the association of  $\gamma$ -casein with the hydrophobic regions of other caseins. It was also observed that, among the different proteose peptones, PP8fast ( $\beta$ -casein: N-28) was released from the casein micelle during hydrolysis much more slowly than PP8slow and PP5 (Figure 66B). This occurred mainly because this  $\beta$ -casein fraction (f1-28) contains a phosphoserine cluster with phosphorylation at four serine residues (as observed in

our results from LC–MS), which are involved in CCP formation; therefore the release of PP8fast (f1–28) was much slower. These observations suggest that CCP and hydrophobic interactions play major roles in maintaining the casein micelle structure against hydrolysis. It is more difficult to comment on accessibility because, even after hydrolysis, the peptides might still be attached to the casein micelle; however, it appears that the N terminus of  $\beta$ -casein (proteose peptones) was more readily accessible to plasmin (as we found that more peptides were released from hydrophilic regions) than the hydrophobic C terminus, forming  $\gamma$ -casein in the casein micelle; similar observations were reported for the trypsin-catalysed hydrolysis of  $\beta$ -casein at an oil–water interface (Leaver & Dalgleish, 1990).

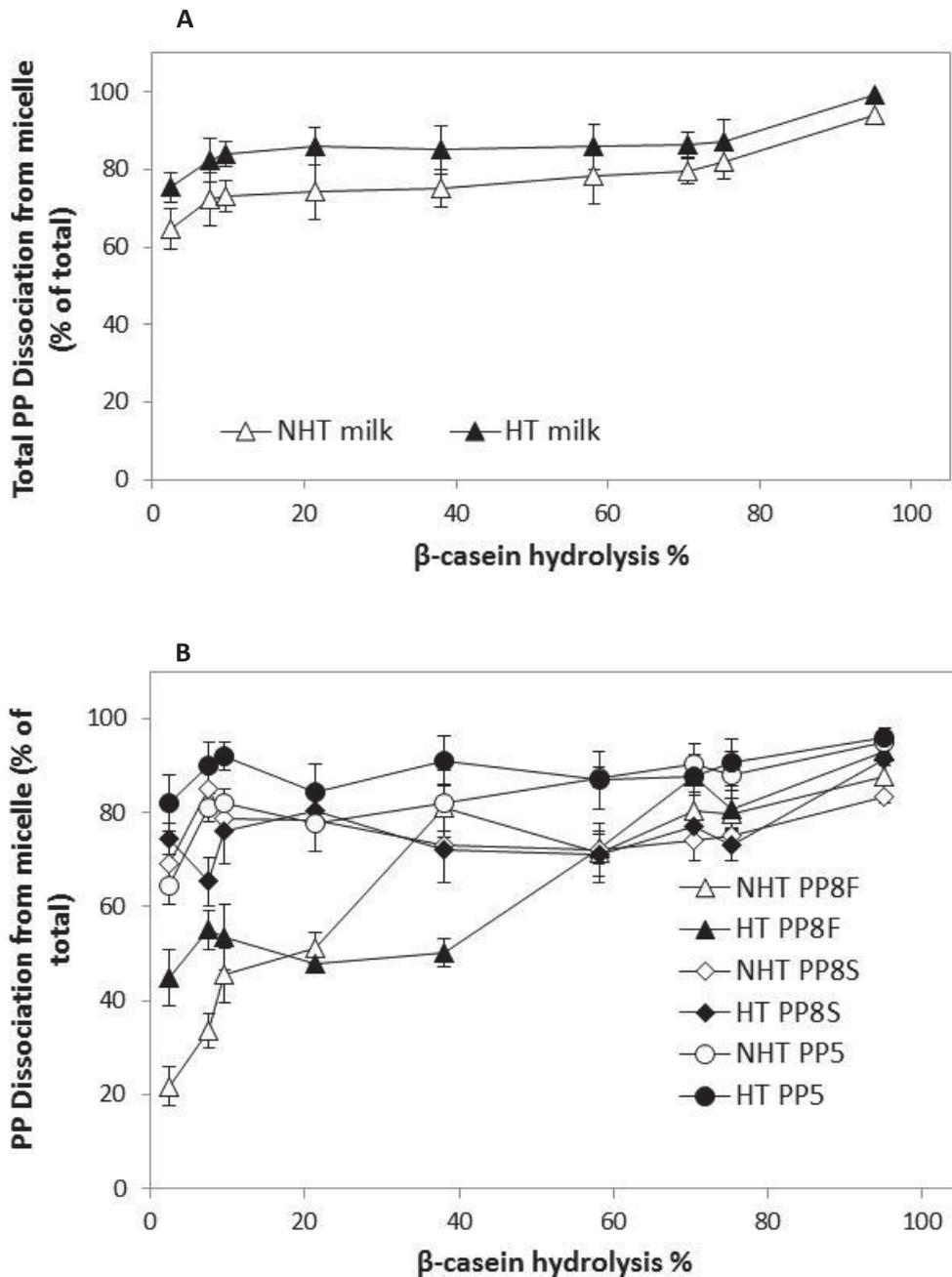


Figure 66. Effect of plasmin-induced hydrolysis on the dissociation patterns of proteose peptones, quantified using RP-HPLC: A, total proteose peptones (PP5 + PP8slow + PP8fast); B, individual proteose peptones. Error bars refer to standard deviations.

Compared with the calculated mesh size of the casein micelle of 4.5 nm (Anema & de Kruif, 2013) and 4.16 nm (Appendix 7), plasmin is a relatively large molecule, with an equivalent spherical diameter of 8.58 nm [based on a Stokes' radius of 4.29 nm determined by Sjöholm, Wiman, and Walléan (1973) for active human plasmin].

Also, according to Anema and de Kruif (2013), the surface of the casein micelle is composed of a layer that is permeable to small molecules/proteins, such as monomeric  $\beta$ -casein or  $\beta$ -lactoglobulin, but not to larger molecules/proteins, suggesting that the plasmin-induced hydrolysis of the casein micelle starts from its surface. The observed higher release of proteose peptones from the micelle from the start of the hydrolysis (Figure 66) is therefore likely to be due mainly to accessibility, suggesting the presence of some  $\beta$ -casein, especially hydrophilic regions, on or close to the surface of the casein micelle. The presence of  $\beta$ -casein near the surface of the casein micelle has been proposed to explain the dissociation behaviour of  $\beta$ -casein during cooling (Creamer et al., 1977; Downey & Murphy, 1970);  $\beta$ -casein has also been theorised to be near the surface, performing a supplementary function of steric stabilisation at low temperatures (de Kruif & Roefs, 1996). This supplementary function of stabilisation by  $\beta$ -casein also explains stabilisation of the casein micelle, given calculations that only one-third is covered by  $\kappa$ -casein (Dalgleish, 1998; Dalgleish et al., 1989), and its behaviour during trypsin-induced hydrolysis (Diaz et al., 1996; Gagnaire & Léonil, 1998; Leaver & Thomson, 1993). As hydrolysis proceeds, plasmin can make its way into the micelle, thereby enabling the hydrolysis of the core of the casein micelle, which consists of plasmin-sensitive  $\alpha$ - and  $\beta$ -caseins; this leads to the casein micelle falling apart, with the slower release of hydrophobic peptides and  $\kappa$ -casein or  $\beta$ -lactoglobulin- $\kappa$ -casein complexes and the faster release of proteose peptones and other hydrophilic peptides. This faster release of hydrophilic peptides from the casein micelle could be the reason for the increased hydrophobicity at limited hydrolysis and the decreased hydrophobicity at excessive hydrolysis (Aroonkamonsri, 1996).

Throughout the hydrolysis, the dissociation of proteose peptone was much greater in heat-treated samples than in non-heat-treated samples. In the heat-treated samples,  $\beta$ -lactoglobulin was associated with the  $\kappa$ -casein on the casein micelle surface but this did not appear to have any hindrance effect on the release of proteose peptones. Again, this phenomenon can be explained by considering the relatively smaller size of these peptides compared with the casein mesh size. The greater dissociation of proteose peptones in the heat-treated milk could have been

due to heat-induced changes in the milk system. Proteose peptones contain all of the phosphoserine clusters in  $\beta$ -casein and thus play a role in the association of  $\beta$ -casein with CCP. High heat treatment of skim milk could cause dephosphorylation (Belec & Jenness, 1962), which would probably result in a heat-induced dissociation of  $\alpha_{s1}$ -,  $\alpha_{s2}$ - and  $\beta$ -caseins because of a weakening of intra-micellar protein–protein interactions (Dalglish et al., 1987). However, Aoki, Umeda, and Kako (1990) observed a weakening of casein–calcium phosphate linkages after heating at 135–140°C for 75 s, even though there was no measurable dephosphorylation of the caseins; instead, they suggested that the transformation of CCP to another form was responsible for the cleavage between CCP and casein on heating milk at high temperatures. Such changes in the form of the CCP could be the main reason for the increased dissociation of proteose peptones in the heat-treated milk upon plasmin-induced hydrolysis (Figure 66). Aoki and Imamura (1975) found that the amount of casein that was solubilised by cooling milk to 4°C was increased markedly by first heating to a high temperature. Similarly, Ward, Goddard, Augustin, and McKinnon (1997) observed that ethylenediaminetetraacetic acid induced increased dissociation of  $\beta$ -casein from the casein micelle in high-heat-treated skim milk and attributed this to the altered structural role of phosphorus but not of calcium in the maintenance of micelle integrity. Nuclear magnetic resonance measurements showed an increase in the mobility of protons on the amino acid side chains of the casein (Rollema & Brinkhuis, 1989); also, the decrease in the solubility of calcium phosphate changes the status or nature of the CCP and could cause weakening of the bonds (Horne, 1998).

#### **8.4.7 Mineral analysis**

CCP plays a critical role in maintaining the integrity of the casein micelle (Holt, Davies, & Law, 1986). With an increase in plasmin hydrolysis, the total calcium and phosphorus content in the serum phase slowly increased (Figure 67), suggesting that the disintegration of the micellar structure was due, in part, to release of CCP containing hydrophilic peptides or nanoclusters, in agreement with the decrease in turbidity (Figure 60), the decrease in particle size (Figure 61) and the increase in proteose peptones in the serum phase (Figure 66). Similar observations on the

release of colloidal minerals into solution were made in experiments in which the casein micelle was disrupted through trypsin hydrolysis (Gagnaire & Léonil, 1998); in contrast, Diaz et al. (1996) did not observe the release of peptides containing phosphoserines, although this could have been due to limited trypsinolysis in their study.

CCP nanoclusters are dispersed as very small (about 2 nm) ‘cherry stones’ in the homogeneous matrix of caseins that form the casein micelle (de Kruif et al., 2012). As discussed above, plasmin-induced hydrolysis starts from the surface of the casein micelle and then moves to the core of the micelle; in this process, the micellar structure opens up and the micelle starts to fall apart, which in turn could possibly allow CCP nanoclusters complexed with hydrolysed peptides to move from the micelle and into the serum phase, thus leading to the observed increase in the total calcium and phosphorus content in the serum phase.

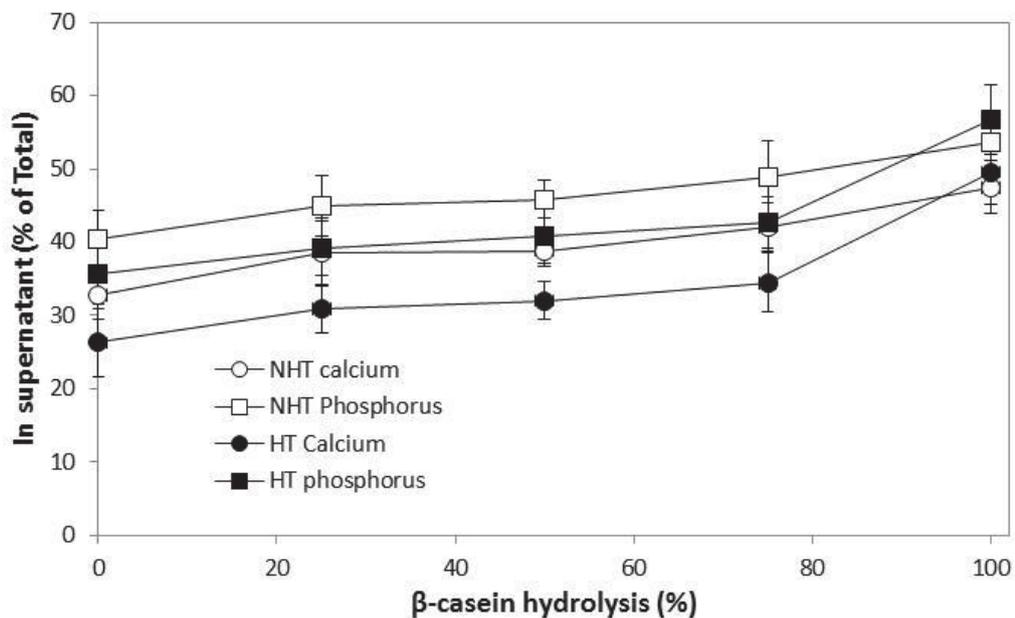


Figure 67. Effect of plasmin-induced hydrolysis on minerals in the serum phase. Error bars refer to standard deviations.

The heat-treated sample had less total calcium and phosphorus in the serum phase (Figure 67) because the solubility of calcium and phosphate decreases with increasing temperature (Anema, 2009a; Holt, 1995); also, heat-precipitated calcium phosphate in milk is protected against sedimentation, probably through association

with the casein micelles (Fox, 1981a). This association of calcium phosphate with the casein micelle in milk has been confirmed using  $^{43}\text{Ca}$  and  $^{31}\text{P}$  nuclear magnetic resonance (Wahlgren, Dejmek, & Drakenberg, 1990). The serum phase calcium and phosphorus concentration increased linearly with increasing  $\beta$ -casein hydrolysis up to 80%. Beyond 80% hydrolysis, there was a steep increase in the serum phase calcium and phosphorus content. From the data collected in this study, it was not possible to determine the exact level of hydrolysis at which the serum phase calcium and phosphorus departed from the linearity exhibited at < 80% hydrolysis. As the range that encompassed the dramatic increase in serum calcium and phosphorus coincided with the region discussed earlier, it seems likely that the increase was also related to the increase in porosity of the micelle that was caused by plasmin-induced dissociation. Interestingly, the serum phase calcium and phosphorus content was lower up to 80%  $\beta$ -casein hydrolysis in the heat-treated milk but became similar to that in the non-heat-treated milk beyond 80% (Figure 67), suggesting dissociation of heat-induced precipitated calcium and phosphate into the serum phase.

It is important to note that, even after 80%  $\beta$ -casein hydrolysis, the particle size was not markedly decreased ( $\sim 20$  nm decrease) and not all the colloidal minerals were released from the micelle, suggesting that part of the structure of the casein micelle, approximately 40% of the  $\kappa$ -casein, 40% of the  $\gamma$ -casein, 20% of the proteose peptones, 20% of the  $\beta$ -casein, 20% of the  $\alpha_s$ -casein and some peptides, was still held together through hydrophobic interactions and CCP.

#### **8.4.8 Sedimentation and SDS-PAGE**

Non-heat-treated and heat-treated milk samples were observed visually for sediment formation under gravity in the tubes. Sediment formation occurred only beyond 80% hydrolysis of  $\beta$ -casein. As shown in Figure 68, the heat-treated samples exhibited significantly more sedimentation than the non-heat-treated samples, which showed hardly any visible sedimentation.

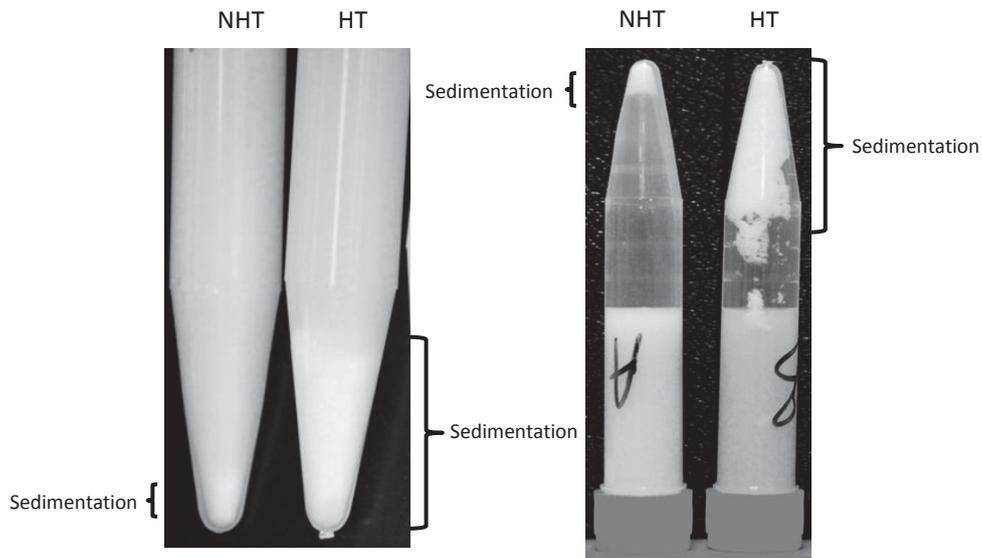


Figure 68. Effect of plasmin-induced hydrolysis on sedimentation in heat-treated (HT) and non-heat-treated (NHT) milk samples.

The protein composition of the sediment in the heat-treated samples was analysed using SDS-PAGE. The major contributing proteins in the sediment were  $\gamma_1$ -,  $\gamma_{2+3}$ - and  $\lambda$ -caseins whereas minor proteins were  $\beta$ -lactoglobulin,  $\kappa$ -casein and traces of  $\alpha_5$ - and  $\beta$ -caseins (Figure 69).

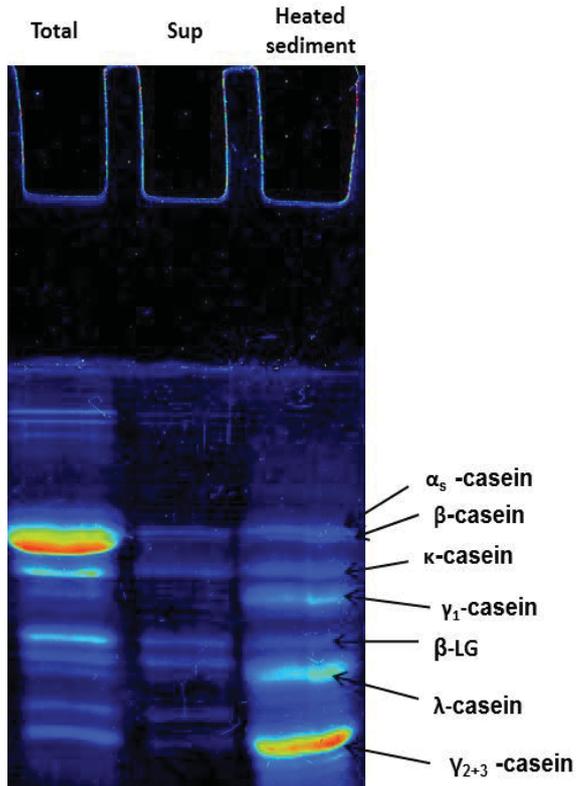


Figure 69. Composition of the sediment as analysed using SDS-PAGE [Total = whole non-centrifuged heat-treated (HT) milk; Sup = supernatant from HT milk; Heated sediment = sediment from HT milk].

The sediment in the heat-treated sample could be attributed mainly to the heat-induced association of  $\beta$ -lactoglobulin with  $\kappa$ -casein, leading to the formation of  $\beta$ -lactoglobulin- $\kappa$ -casein complexes on the micelle surface (Guyomarc'h et al., 2003; Jang & Swaisgood, 1990; Singh & Fox, 1987a).  $\beta$ -Casein and  $\alpha_s$ -caseins form the anchoring points for the  $\kappa$ -casein in non-heat-treated milk and for the  $\beta$ -lactoglobulin- $\kappa$ -casein complexes in heat-treated milk. Plasmin-induced hydrolysis of  $\beta$ -casein and  $\alpha_s$ -caseins leads to the release of  $\kappa$ -casein or  $\beta$ -lactoglobulin- $\kappa$ -casein complexes from the casein micelle, as observed by SDS-PAGE analysis (Figure 65B). Crudden et al. (2005b) also observed a greater increase in the level of non-sedimentable  $\kappa$ -casein when milk was stored at either 5 or 37°C in the presence of plasmin.

According to the age gelation mechanism proposed by McMahon (1996), age gelation is initiated by the release of  $\beta$ -lactoglobulin- $\kappa$ -casein complexes, formed during heating, into the milk serum, possibly by plasmin hydrolysis around 'anchor

points'. The released  $\beta$ -lactoglobulin- $\kappa$ -casein complexes eventually form a protein network and thus form a gel (Datta & Deeth, 2001). In the first stage, proteases cleave the peptide bonds that anchor  $\kappa$ -casein to the casein micelle and facilitate the release of  $\beta$ -lactoglobulin- $\kappa$ -casein complexes. The second stage involves the aggregation of  $\beta$ -lactoglobulin- $\kappa$ -casein complexes and the formation of a three-dimensional network of cross-linked proteins (Datta & Deeth, 2001; Manji & Kakuda, 1988). Many researchers (Kohlmann et al., 1991; Rauh et al., 2014a; Venkatachalam et al., 1993) have commented on a possible role of plasmin in the age gelation of UHT milk. According to our results, proteose peptones were released from the micelle first whereas the release of  $\gamma$ -casein and  $\kappa$ -casein was much slower (Figure 65), which means that, even after hydrolysis by plasmin, the release of  $\beta$ -lactoglobulin- $\kappa$ -casein complexes from the casein micelle was too slow [ $\sim 60\%$  release at  $80\%$   $\beta$ -casein hydrolysis (Figure 65B)] to cause any gelation. This is in agreement with the observation of Rauh et al. (2014a), who reported a low concentration of  $\beta$ -lactoglobulin and  $\kappa$ -casein in gel formation. Beyond  $80\%$  hydrolysis, most of the casein micelles became hollow and dissociated to form a system that contained mostly peptides, which may not have been able to form a gel. The literature is divided on the possibility of gel formation in systems with high levels of  $\beta$ -casein hydrolysis: Kelly and Foley (1997) and Newstead et al. (2006) did not observe any gelation in their systems, with complete breakdown of  $\beta$ -casein, whereas Rauh et al. (2014a) reported gelation even after  $95\%$   $\beta$ -casein  $A^1$  hydrolysis. We observed that the  $\beta$ -lactoglobulin- $\kappa$ -casein complexes released from the micelle by plasmin-induced hydrolysis in heat-treated milk systems did not form a gel; instead, they were involved in the formation of large aggregates through hydrophobic interactions with other  $\beta$ -lactoglobulin- $\kappa$ -casein complexes to a minor degree but mostly with hydrophobic peptides, e.g.  $\gamma$ -casein, that were formed and released through plasmin-induced hydrolysis. These larger aggregates then settled rapidly at the bottom of the container, resulting in the sediments observed (Figure 68) in the heat-treated milk systems. Aggregates suspended in the serum phase could have been the main reason for the increased turbidity and the increased particle size beyond  $80\%$  hydrolysis of  $\beta$ -casein. The absence of  $\beta$ -lactoglobulin- $\kappa$ -casein complexes in the non-heat-treated milks led to hardly any sedimentation.

Our observations on sediment formation as a result of plasmin-induced hydrolysis are in agreement with Enright, Bland, Needs, and Kelly (1999), who observed that raw milk and UHT samples into which either  $\text{KIO}_3$  or plasmin was added possessed sediments that consisted of large densely packed aggregates of extensively linked micelle-like particles.

In the present study, as plasmin was added externally at a higher level than that present in milk to facilitate rapid hydrolysis, our system did not show any storage-induced physicochemical changes and there was no gel formation. For age gelation in UHT milk, the level of plasmin is lower and gelation takes place on prolonged storage, during which storage-induced physicochemical changes such as Maillard reactions, ionic calcium and changes in pH occur (Andrews & Cheeseman, 1972; Kelly & Foley, 1997; Venkatachalam et al., 1993). When interpreted in the light of our findings, it would appear that these storage-induced physicochemical changes are necessary and therefore play an important role in gel formation in association with low levels of plasmin-induced hydrolysis (Kelly & Foley, 1997; Kohlmann et al., 1991; Manji & Kakuda, 1988). Interestingly, according to some studies (Manji et al., 1986; Zadow & Chituta, 1975), storage at higher temperature ( $> 35^\circ\text{C}$ ) may retard gelation, which again indicates the importance of hydrophobic interactions, because hydrophobic interactions are stronger at high temperatures and thus hold the micelle together and prevent dissociation (Venkatachalam et al., 1993).

## 8.5 CONCLUSIONS

The present study explains the plasmin-induced dissociation of the casein micelle in relation to the release of different peptides from the casein micelle along with the physicochemical changes in both heat-treated and non-heat-treated milk systems. Particle size and turbidity results indicated that casein micelle dissociation occurred during the early stages and that aggregation of hydrolysed peptides occurred towards the final stages of hydrolysis. Hydrolysis results indicated that hydrophilic peptides, e.g. proteose peptones, were the first to dissociate from the casein micelle on plasmin-induced hydrolysis. In contrast, hydrophobic peptides, e.g.  $\gamma$ -caseins, dissociated slowly and with dissociation patterns that were identical to

those of  $\kappa$ -casein, suggesting that, even after breakage of the anchor points, the release of  $\kappa$ -casein from the micelle was too slow to cause gelation. The hydrolysis pattern was also found to change with the heat treatment. The heat-treated milk system was found to have greater dissociation from the casein micelle of both hydrophobic peptides (e.g.  $\gamma$ -caseins) and hydrophilic peptides (e.g. proteose peptones). There was no sign of gelation in either system. Heat-treated samples had clearly visible sedimentation whereas no sedimentation was apparent in non-heated samples. Therefore, it was concluded that heat-induced association of whey proteins with the casein micelle followed by plasmin-induced hydrolysis resulted in increased sedimentation levels. These results provide new insights into the dissociation pattern of the casein micelle and further insights into the importance of the role of plasmin in sediment formation in heat-treated milk and beverage systems.

To achieve further understanding on the mechanism of plasmin-induced age gelation, a similar study with a prolonged post-UHT storage period is needed, along with the identification of the peptides released at different levels of hydrolysis and their relation to bitterness and concomitant changes in the casein micelle structure.



## Chapter 9: Overall conclusions

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The aim of this thesis was to understand and control the plasmin-induced hydrolysis of caseins in milk systems. A sequential approach was used. Firstly, plasmin-induced hydrolysis of a pure single-protein model system (using  $\beta$ -casein) was studied. Modification of the lysine residue resulted in a decrease in the availability of the substrate for plasmin, which led to the development of a control mechanism utilising protein modification. In the second stage, based on this mechanism, different protein modifications were applied to a real milk system to minimise plasmin-induced hydrolysis. Analysis of the real milk system included consideration of the casein micelle structure, whey protein denaturation and association with the casein micelle, and system complexity. The final stage of the study investigated the plasmin-induced dissociation of casein micelles in real milk systems to understand the effect on gelation and sedimentation in heat-treated milk.

The main objective of the first experimental section of the study was to understand and control the plasmin-induced hydrolysis of a pure protein ( $\beta$ -casein) model system.  $\beta$ -Casein was used as a model system, because it is the primary target for plasmin-induced hydrolysis in milk systems. This objective was achieved and a mechanism for the prevention of plasmin's action on proteins was established. A systematic study was carried out, starting with a simple chemical modification of proteins at lysine residues, i.e. succinylation, and progressing to more food-grade and complex approaches of protein modification, i.e. lactosylation and transglutamination. As modification of the  $\epsilon$ -amino group of the lysine residue of  $\beta$ -casein made it unrecognisable to the substrate-binding pocket of plasmin, the substrate was not positioned into the pocket and could not be hydrolysed by the catalytic triad of plasmin, thus decreasing the hydrolysis of  $\beta$ -casein by plasmin. As confirmed by liquid chromatography–tandem mass spectrometry analysis and by identification of succinylation sites, the substrate was modified mainly by the involvement of the lysine residue in the succinylation reaction, resulting in charge-related and steric-availability-related conformational changes in  $\beta$ -casein. The same

mechanism could be applied for lysine modification through food-grade reactions occurring during heat treatment, i.e. lactosylation. However, because of the complexity of the Maillard reaction and to understand and explain the mechanism of the resistance of protein to plasmin, these three different means of substrate modification were compared. This comparison indicated that lysine modification played a major role in minimising plasmin-induced hydrolysis, in comparison with cross-linking. Lactosylation was found to have a greater effect than succinylation at the same level of lysine modification. A mechanism for this phenomenon was proposed. Lactosylation involves the attachment of lactose and, in advanced stages, cross-linking, thus modifying lysine and making it unrecognisable to plasmin; in addition, the cross-linking may affect the release of plasmin-generated peptides. Transglutamination also modifies lysine by cross-linking and has a similar effect to lactosylation, but to a lesser extent. In contrast, succinylation modifies the charge associated with lysine, making it unrecognisable to plasmin. These results explain the different effects of each type of substrate modification and this knowledge can be used to make protein resistant to plasmin-induced hydrolysis.

The objective of the second experimental section of the thesis was to apply the approach and mechanism developed to inhibit plasmin-induced hydrolysis in the first section on a pure protein model system to the more complex real milk system containing casein micelles, to understand the effect of the casein micelle structure and its changes on plasmin-induced hydrolysis.

The proteins were modified using succinylation and the succinylation sites on the different proteins were successfully identified as: Lys7, Lys34, Lys36, Lys42, Lys83 and Lys124 in  $\alpha_{s1}$ -casein; Lys80, Lys150, Lys152, Lys158 and Lys165 in  $\alpha_{s2}$ -casein; Lys28, Lys29, Lys32, Lys99, Lys105, Lys107 and Lys113 in  $\beta$ -casein; Lys75, Lys77, Lys83, Lys91, Lys100, Lys135 and Lys138 in  $\beta$ -lactoglobulin. In agreement with the previous work on protein modification, the succinylation of skim milk decreased the plasmin-induced hydrolysis; however, the increased negative charges on the caseins led to dissociation of the casein micelle, exposing the caseins to plasmin's action. As a result of this casein micelle dissociation, the trend was non-linear and different from that in the previous pure  $\beta$ -casein study: at low levels of succinylation, the

decrease in the hydrolysis rate was linear because of the substrate modification; however, at higher levels of succinylation, it was the dissociation of the caseins from the micelle that dominated, resulting in an increase in the micelle mesh size (porosity), which reduced steric hindrance, making plasmin-sensitive proteins readily available for hydrolysis and the negative effect became less significant. It was concluded from the study on the succinylation of skim milk and its effect on plasmin-induced hydrolysis that the casein micelle structure plays an important role in protecting caseins against plasmin's action.

The effect of lactosylation and whey protein association with the casein micelle on plasmin-induced hydrolysis in skim milk was investigated, as high-heat-treated (120°C/15 min) skim milk had been found to have higher resistance to plasmin-induced hydrolysis than a non-heated skim milk. Both whey protein association with the casein micelles and lactosylation decreased the availability of protein to plasmin. These effects were compared and mechanisms were proposed. Whey-protein-free milk was the most resistant to plasmin, followed by skim milk and lactose-free milk, suggesting that lactosylation plays a more major role than whey protein association with the casein micelles in making protein resistant to plasmin-induced hydrolysis. The present study can be useful in providing a method to control the plasmin activity in milk systems that are intended to be high heat treated. The lactosylation level and the whey protein association with the casein micelle can be tailored by tuning the processing parameters and thus plasmin-induced hydrolysis can be controlled in real milk systems.

The objective of the third experimental section was to understand plasmin-induced dissociation of the casein micelle and physicochemical changes in milk due to plasmin's action, to give further insights into the role of plasmin in sedimentation and gelation in milk. The study explains the plasmin-induced dissociation of the casein micelle in relation to the release of different peptides from the casein micelle along with the physicochemical changes in both heat-treated and non-heat-treated milk systems. Particle size and turbidity decreased during the initial hydrolysis but increased dramatically towards the end of hydrolysis. This indicated that casein micelle dissociation occurred during the early stages and that aggregation of

hydrolysed peptides occurred towards the final stages of hydrolysis. The total calcium and phosphorus level in the serum phase increased linearly with an increase in the extent of hydrolysis, suggesting the release of peptides containing colloidal calcium phosphate from the casein micelle. Hydrolysis results indicated that hydrophilic peptides, e.g. proteose peptones, were the first to dissociate from the casein micelle on plasmin-induced hydrolysis. In contrast, hydrophobic peptides, e.g.  $\gamma$ -caseins, dissociated slowly and with dissociation patterns that were identical to those of  $\kappa$ -casein, suggesting that, even after breakage of the anchor points, the release of  $\kappa$ -casein from the micelle was too slow to cause gelation. The hydrolysis pattern was also found to change with the heat treatment. The heat-treated milk system was found to have greater dissociation from the casein micelle of both hydrophobic peptides (e.g.  $\gamma$ -caseins) and hydrophilic peptides (e.g. proteose peptones). The particle size and the turbidity were also higher in heat-treated samples than in control samples at the end of the hydrolysis. There was no sign of gelation in either system. Heat-treated samples had clearly visible sedimentation whereas no sedimentation was apparent in non-heated samples. Therefore, it was concluded that heat-induced association of whey proteins with the casein micelle followed by plasmin-induced hydrolysis resulted in increased sedimentation levels. These results provide new insights into the dissociation pattern of the casein micelle and further insights into the importance of the role of plasmin in sediment formation in heat-treated milk and beverage systems. To achieve further understanding of the mechanism of plasmin-induced age gelation, a similar study with a prolonged post-UHT storage period is needed, along with the identification of the peptides released at different levels of hydrolysis and their relation to bitterness and the concomitant changes in the casein micelle structure.

## Chapter 10: Future directions

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The present study showed that substrate modification, together with the developed understanding of the dissociation of the casein micelle upon plasmin-induced hydrolysis, can be useful in controlling the plasmin-induced hydrolysis of caseins. It was suggested that further research work is needed to understand the mechanism of plasmin inhibition by substrate modification and to understand casein micelle dissociation during storage with reference to sedimentation and age gelation.

1. A study of the crystal structure of bovine plasmin is needed to support the proposed mechanism of plasmin inhibition using substrate modification, which is based on sequence homology with human plasmin. Identification of substrate-binding-pocket residues will improve our understanding of the control of plasmin activity. The mechanism of plasmin's selectivity or substrate specificity and plasmin's preference for certain substrates or residues can be further studied with reference to the known three-dimensional molecular structures of different milk proteins.
2. Lactosylation of the substrate was found to have a negative effect on plasmin-induced hydrolysis. As plasmin and its zymogen (plasminogen) are indigenous to milk, any high heat treatment could possibly cause lactosylation of plasmin and plasminogen. Lactosylation-induced structural changes in the globular enzymes and cross-linking of residues could have an effect on the enzyme's activity and the zymogen's activation. A study on the effect of the lactosylation of plasmin (and plasminogen) on its structure and activity will improve understanding. A hypothesis around the glycation of plasmin in diabetic patients could be built.
3. In the present study, the effect of plasmin-induced hydrolysis on casein micelle dissociation was investigated with the elimination of storage-induced changes in the system by adding plasmin at higher concentrations; this can be used as a basis for further understanding. Plasmin-induced hydrolysis in combination with

and separately from the long-term storage-induced physicochemical changes is needed to understand the real life scenario of age gelation or sedimentation.

4. Plasmin's activity at interfaces and its effect on colloidal/emulsion stability together with its effect on interfacial and functional properties in dairy emulsions/colloidal systems could produce useful results and understanding for beverage and cream applications.
5. A study of plasmin activity along with lipase activity in dairy emulsion systems could be useful for an understanding of the issues related to bitterness and instability in these systems.

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# Appendices

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## Appendix 1. Effect of calcium chelation on plasmin-induced hydrolysis

### A. Effect of calcium chelation on casein micelle structure

With an increase in the ethylenediaminetetraacetic acid (EDTA) concentration, the transparency of the skim milk samples (Figure 70) and the measured turbidity decreased linearly, indicating dissociation of the casein micelles in the milk system.

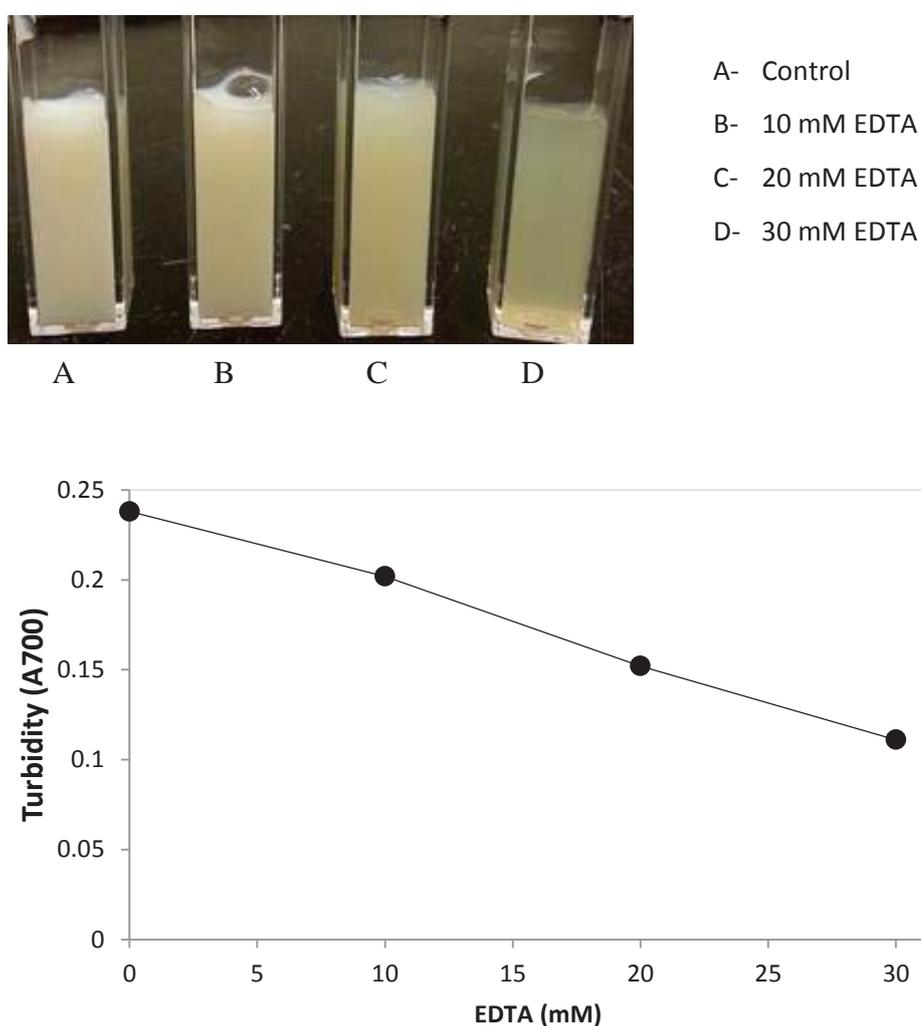


Figure 70. Effect of EDTA addition on (A) visual changes and (B) turbidity of skim milk.

Changes in particle size with an increase in the EDTA concentration were measured. There was not much change in the particle size at up to 20 mM EDTA addition; however, it decreased markedly at 30 mM EDTA addition, whereas the mean count

rate decreased continuously (Figure 71A), in agreement with the turbidity observations. The decreases in turbidity, particle size and mean count rate were due to chelation of colloidal calcium by EDTA, resulting in dissociation of the micelle as a consequence of disruption of the colloidal calcium phosphate. The minimal change in the particle size up to 20 mM EDTA addition could have been due mainly to the increase in the negative charges on the caseins because of the calcium chelation, leading to increased repulsion among the caseins in the micelle and swelling of the casein micelle. Thus, the intra-micellar repulsion hinders the dissociation of caseins from the micelle, resulting in minimal change in the particle size; however, the micelle becomes hollow, as reflected in the turbidity and mean count rate results.

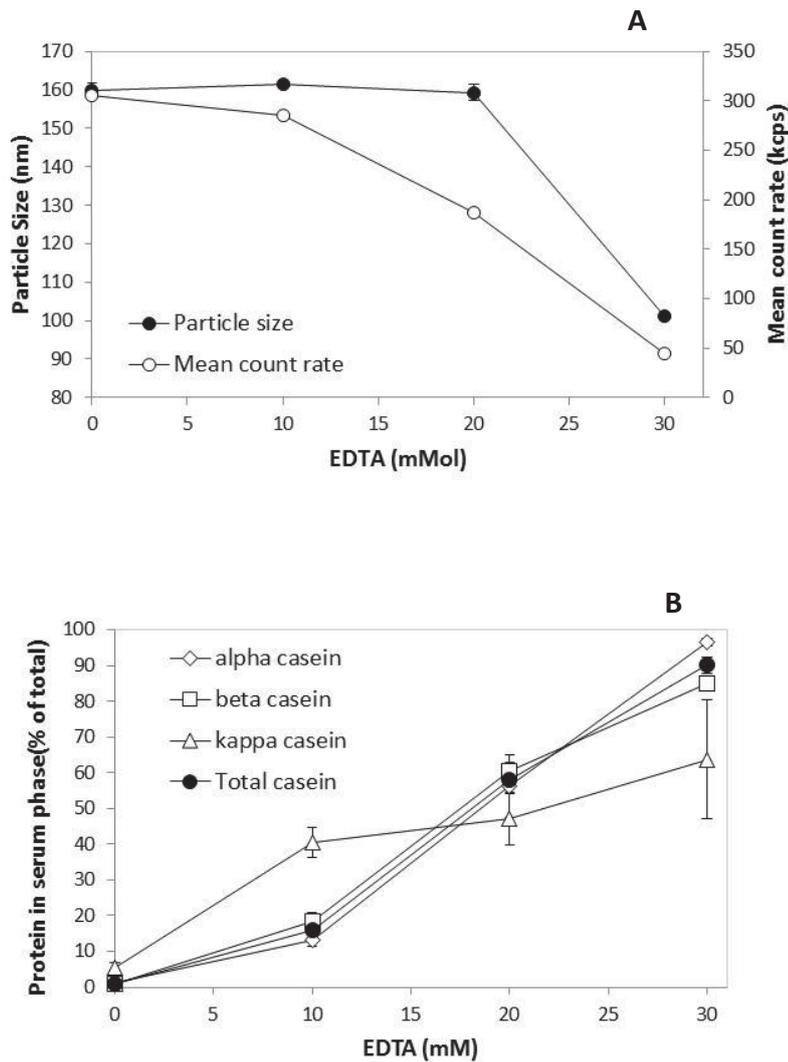


Figure 71. Effect of EDTA addition on changes in (A) particle size and (B) dissociation of proteins from the casein micelle in skim milk.

Micelle dissociation was measured using a Bio-analyser (Anema, 2009b) and was found to increase continuously with increasing EDTA concentration (Figure 71B). Among the caseins, the dissociation of  $\kappa$ -casein increased markedly to 40% at 10 mM EDTA; the increase then slowed with further increases in EDTA, with 50% dissociation at an EDTA concentration of 20 mM. In contrast, the dissociations of  $\alpha$ -casein,  $\beta$ -casein and total caseins were less at 10 mM EDTA but increased continuously with an increase in the EDTA concentration (Figure 71B).

## B. Effect of casein micelle dissociation on plasmin-induced hydrolysis

Plasmin-induced hydrolysis increased with an increase in the micelle dissociation caused by EDTA addition, as measured by pH-4.6-soluble peptides and 6%-trichloroacetic acid (TCA)-soluble peptides.

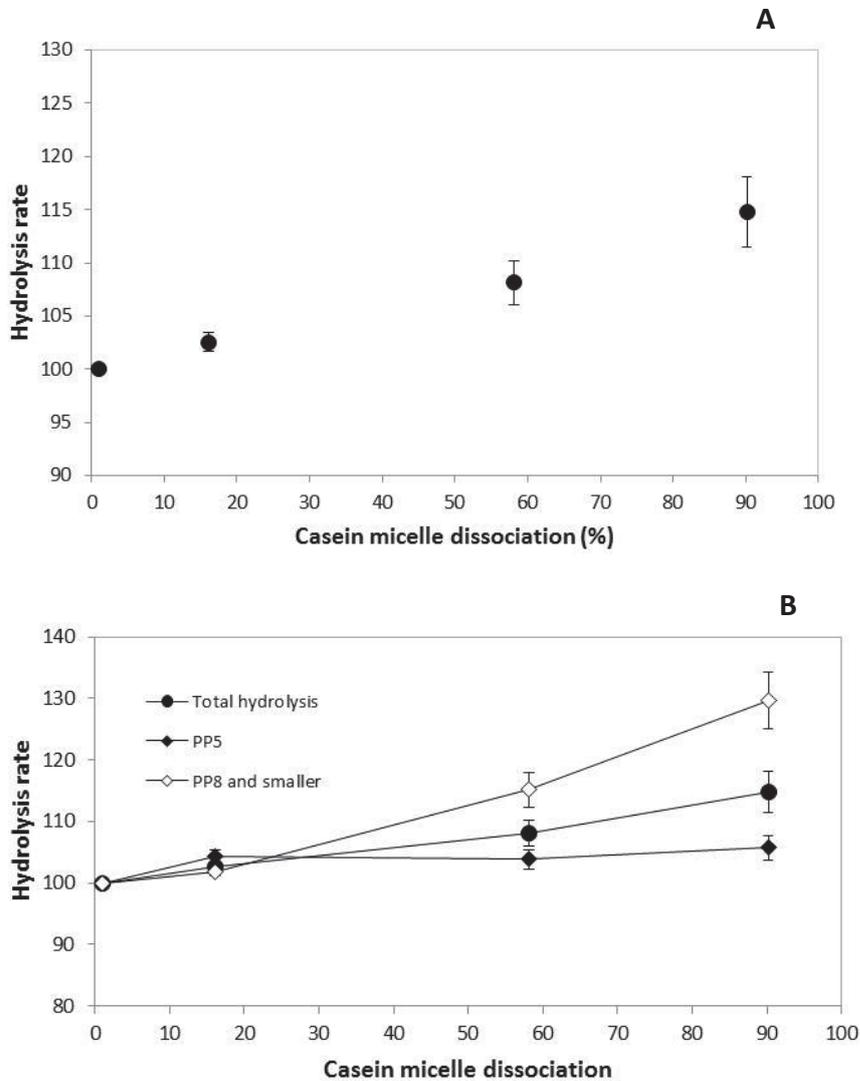


Figure 72. Effect of casein micelle dissociation on the plasmin-induced hydrolysis rate: A, hydrolysis rate measured from the gradient of 6%-TCA-soluble peptides; B, hydrolysis rate measured from the gradient of pH-4.6-soluble peptides.

Plasmin-induced hydrolysis of casein is affected by the structure of the casein micelle. Dissociation of caseins from the casein micelle, caused by EDTA addition, resulted in a decrease in the turbidity and an increase in micelle porosity, which collectively reduced steric hindrance and made the protein more readily hydrolysed by plasmin. The major impact of calcium chelation was on the proteose peptones

PP8slow and PP8fast, indicating increased sensitivity of the bond Lys28–Lys29 upon calcium chelation.

Overall, it can be concluded that the casein micelle structure acts to protect the caseins against plasmin-induced hydrolysis; the involvement of the caseins in hydrophobic and colloidal calcium phosphate interactions and the formation of the complex structure limit the accessibility of plasmin to the caseins.

## Appendix 2. Reaction order of $\beta$ -casein hydrolysis

By the half-life (concentration versus time) method

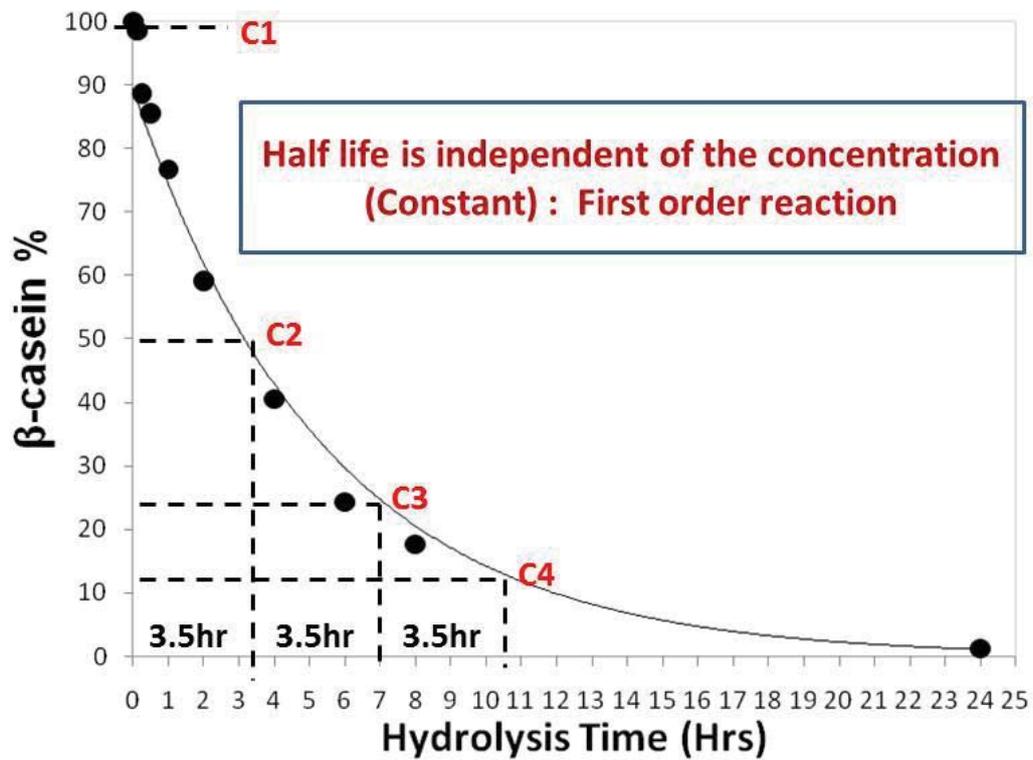


Figure 73. Reaction order of the plasmin-induced hydrolysis of  $\beta$ -casein.

- The plasmin-induced hydrolysis of  $\beta$ -casein is a first order reaction.

**Appendix 3. Identification of  $\gamma$ -casein elution pattern on SDS-PAGE**

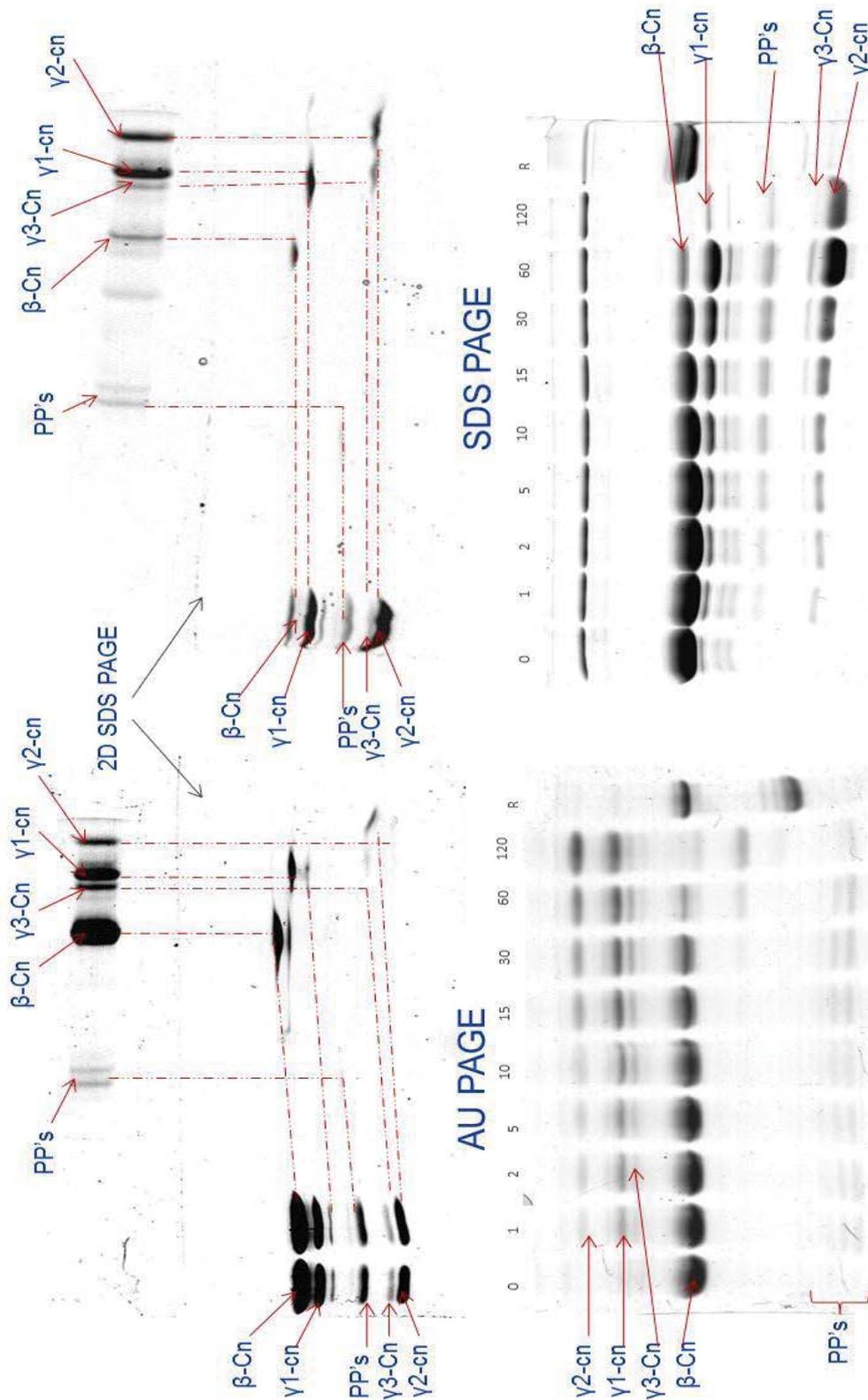


Figure 74. Identification of  $\gamma$ -casein elution pattern on SDS-PAGE using a two-dimensional PAGE technique.

#### Appendix 4. Identification and elution pattern of proteose peptone fractions and hydrolysis analysis using RP-HPLC

Stage 1: Pure PP5 peptide was loaded on to the RP-HPLC system and the elution pattern was identified.

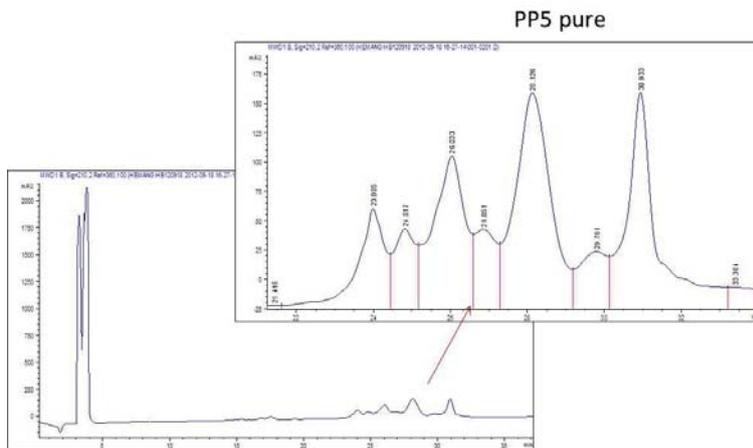


Figure 75. Identification of the elution pattern of PP5 on RP-HPLC.

Stage 2: Pure PP5 was hydrolysed by plasmin to different levels (different incubation times) to generate two major peptides, PP8slow and PP8fast. The elution patterns of these peptides were identified.

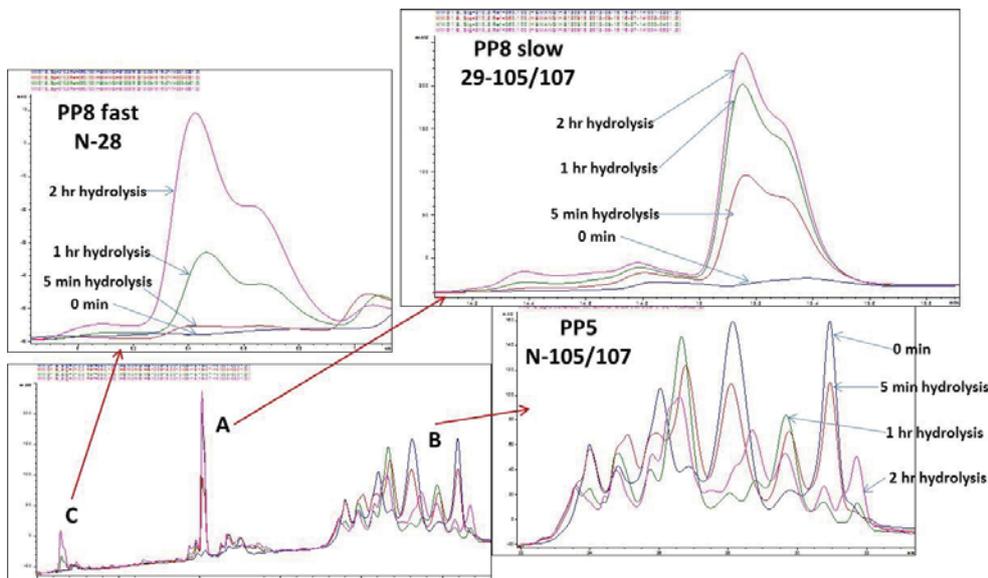


Figure 76. Identification of RP-HPLC elution patterns of different proteose peptones from the plasmin-induced hydrolysis of PP5.

Plasmin-induced hydrolysis analysis was carried out by measuring the hydrolysis products of  $\beta$ -casein using RP-HPLC. Proteose peptones are the hydrolysis products – e.g. PP5,  $\beta$ -casein f(N–105/107); PP8fast,  $\beta$ -casein f(N–28); PP8slow,  $\beta$ -casein f(29–105/107) – of  $\beta$ -casein that are heat stable and acid soluble at pH 4.6 (Andrews, 1978a, 1978b; Andrews & Alichanidis, 1983; Eigel, 1981; Kolar & Brunner, 1969, 1970). pH-4.6-soluble extracts were obtained by lowering the pH to 4.6 and removing the precipitates by centrifugation; the generated hydrolysed peptides were analysed. With an increase in plasmin-induced hydrolysis, the generation of and increases in peptide peaks at different elution times were observed. The quantification was carried out by calculating the areas under different proteose peptone peaks and summing all the peaks (total proteose peptones) using Chemstation software.

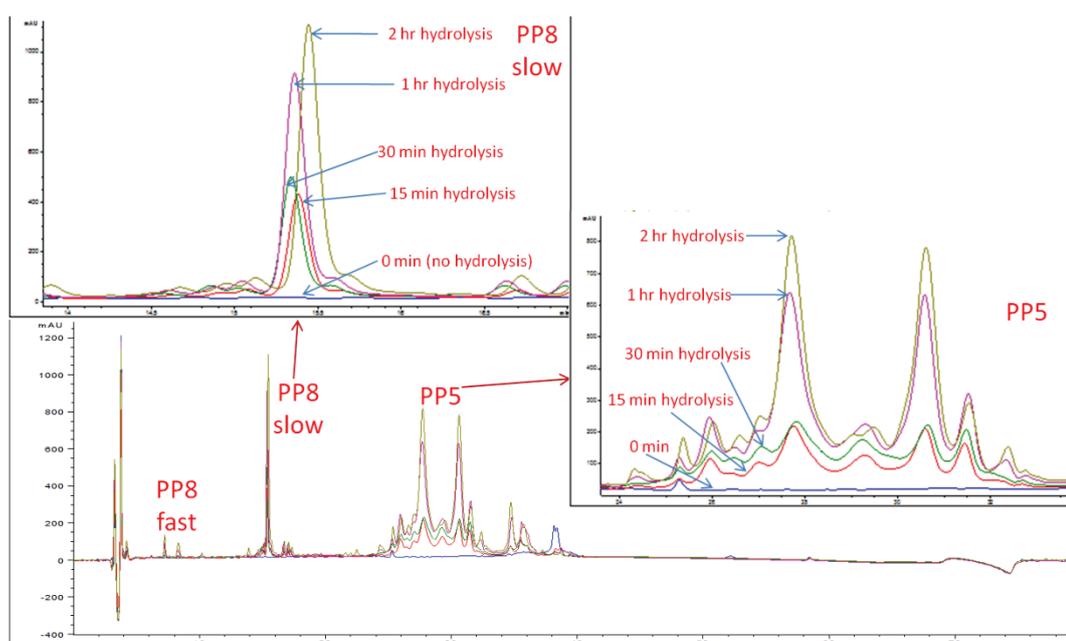


Figure 77. Hydrolysis patterns for control sample (from 0 to 2 h) as measured in pH-4.6-soluble extracts by RP-HPLC.

For the analysis of the hydrolysis, three peaks – PP8slow (at 15 min elution time), PP5 (group of peaks at 24–34 min elution time) and PP8fast (6–9.6 min elution time) – were observed (Figure 77). The PP8slow, PP5 and PP8fast peaks all increased with the hydrolysis time and were clearly correlated with the hydrolysis time. The areas under different proteose peptone peaks were calculated separately

and the sum of all peaks, i.e. total proteose peptone (PP8slow + PP5 + PP8fast), was also calculated.

In the initial stages of hydrolysis, PP5 and PP8slow were the first peaks to be formed and were found to increase rapidly from 0 to 2 h of hydrolysis. PP8fast was not prominent in the initial stages and started to form after 1 h of hydrolysis, which could have been due to the formation of PP8fast from the hydrolysis of PP5.

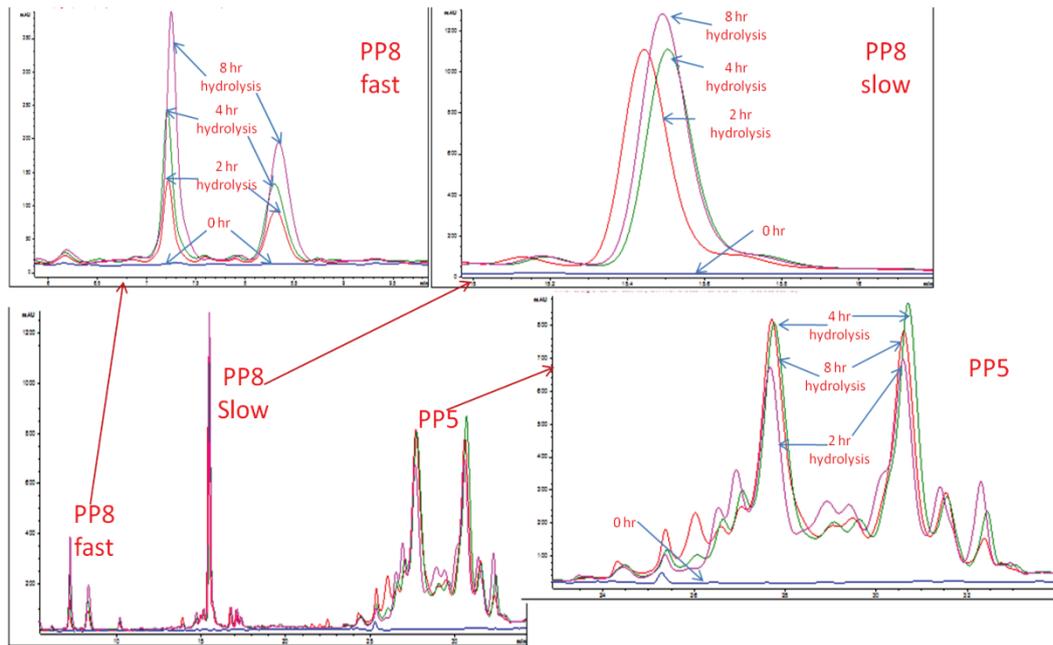


Figure 78. Hydrolysis patterns for control sample (from 0 to 8 h) as measured in pH-4.6-soluble extracts by RP-HPLC.

Upon prolonged hydrolysis up to 8 h, the increases in peaks PP5 and PP8slow were much reduced from 2 to 4 h and from 4 to 8 h, which indicated a decrease in the availability or the unavailability of the substrate ( $\beta$ -casein) generating PP5 and PP8slow. In contrast, PP8fast increased continuously at a very high rate, which indicated that the substrate (PP5) producing PP8slow was available for hydrolysis; this can also be seen as the slow increase in PP8s and the decrease in PP5 from 4 to 8 h of hydrolysis (Figure 78).

## Appendix 5. Heat-induced hydrolysed peptides generated on the high heat treatment of skim milk

Peak formation at an elution time of 5 min increased with an increase in heat treatment in samples before plasmin addition (0 min samples) (Figure 79).

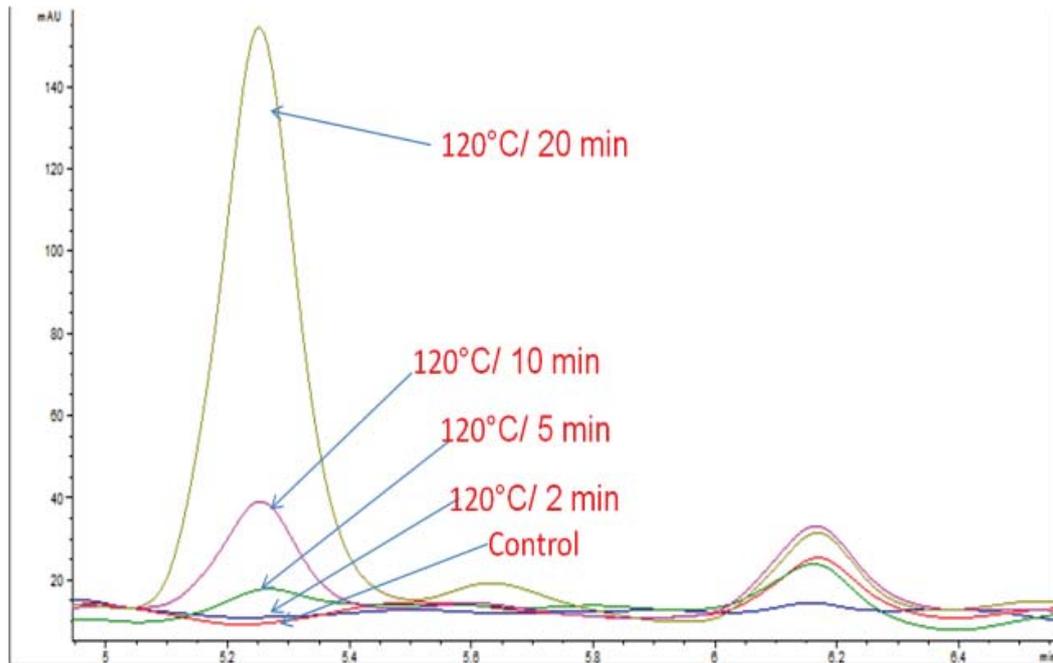


Figure 79. Heat-induced hydrolysed peptide peak after 5 min (as measured by RP-HPLC of pH-4.6-soluble fractions).

This peak formation indicated the heat-induced hydrolysis of  $\beta$ -casein at high temperature, which led to the generation of small peptides, as observed by the early elution in pH-4.6-soluble fractions on RP-HPLC and at less than 1 kDa in 6%-TCA-soluble fractions on SE-HPLC. Although the 6%-TCA-soluble fractions were free of large peptides, the pH-4.6-soluble fractions were not (could contain proteose peptones).

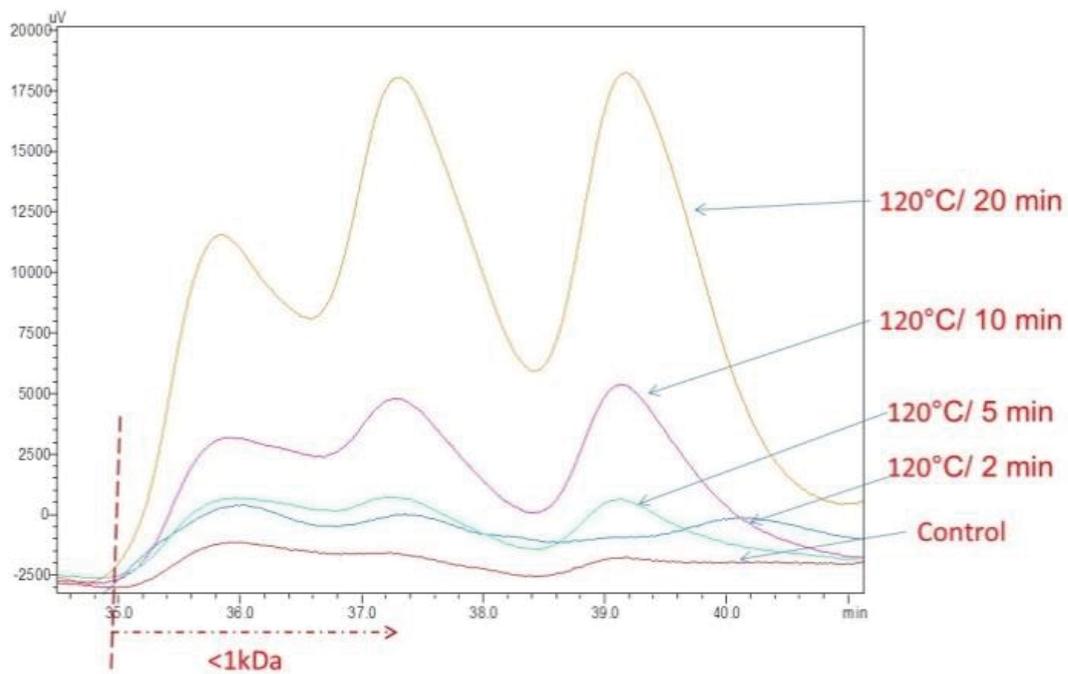


Figure 80. Heat-induced hydrolysed peptide size < 1 kDa (as measured by SE-HPLC of 6%-TCA-soluble fractions).

These heat-induced peptides were in very small concentration compared with the plasmin-hydrolysed products and were not affected or changed during the hydrolysis. Therefore, they were not considered during the plasmin-induced hydrolysis analysis.

## Appendix 6. RP-HPLC–MS/MS analysis for lactosylated peptides

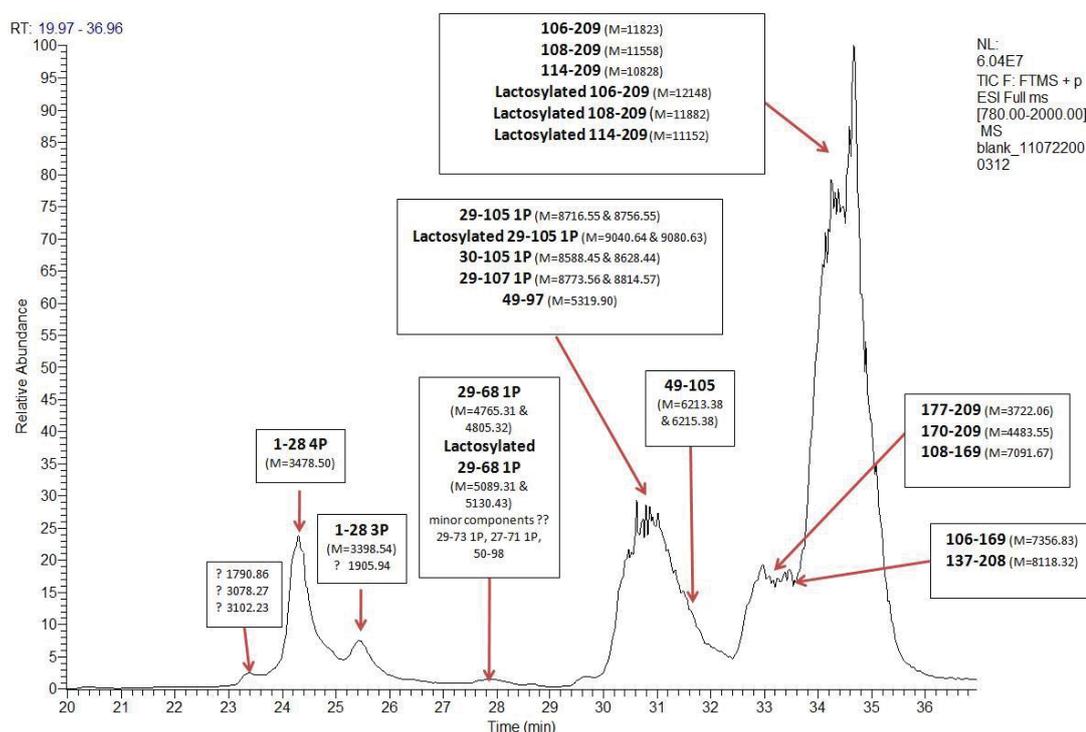


Figure 81. LC–MS elution pattern of lactosylated peptides of  $\beta$ -casein.

Table 18. Estimation of lactosylation sites in peptides of  $\beta$ -casein

No.	Lactosylated peptides:	Lys-X	Lysine residues in chain
1	His106-Val209	Lys105	Lys-107, 113, 169, 176,
2	Glu108-Val209	Lys107	Lys- 113, 169, 176,
3	Tyr114-Val209	Lys113	Lys-169, 176
4	Lys29-Lys105	Lys28,105	Lys-29, 32, 48, 97, 99,
5	Ile30-Lys48	Lys29	Lys-32
6	Glu108-169	Lys107	Lys113
7	Tyr114-Lys176	Lys113	169
8	Ile30-Lys105	Lys29	Lys-32, 48,
9	Lys29-Asn68	Lys28	Lys-29, 32, 37C, 48,

- $\gamma_1$ -Casein is the first peptide to be lactosylated – primarily at Lys107 (Scaloni et al., 2002).
- With an increase in heat treatment, other peptides are also lactosylated.
- The first four are major lactosylated peptides – primary targets (see Table 18).
- Lactosylation of  $\gamma_2$ -casein – stable against plasmin hydrolysis.
- Lactosylated peptides (see Table 18): 120°C/5 min – 1 and 120°C/10 min – 1–9.

## Appendix 7. Calculation of the mesh size of the casein micelle

If  $r = 100$  nm is the radius of a casein micelle of 200 nm diameter, then

volume of the micelle =  $\frac{4}{3}\pi r^3$  where  $r = 100$  nm

$$V = 4188790.20 \text{ nm}^3 \quad \dots\dots\dots\text{eq. 1}$$

Here, the micelle is a sphere. For simplicity of the calculations, a cube of the same volume having a side of length 'a' was assumed; later, the same cube made up of smaller cubes of side 'a<sub>s</sub>' was assumed.

Volume of the sphere = volume of the cube

$$\frac{4}{3}\pi r^3 = a^3$$

$$a^3 = 4188790.20$$

$$a = 161.19 \quad \dots\dots\dots\text{eq. 2}$$

Now,

$$\text{Mass} = V \cdot \frac{\rho}{4.4} \quad \dots\dots\dots \text{(here } \rho = 1016 \frac{\text{kg}}{\text{m}^3} \text{ and for casein } \frac{1}{4.4} \text{) .. eq. 3}$$

Therefore, from eq. 1 and eq. 3

Mass = 4188790.205 x 1016 x 10<sup>-27</sup> (to convert nanometres into kilograms, we need to multiply by 10<sup>-27</sup>)

$$\text{Mass} = 9.67 \times 10^{-19} \quad \dots\dots\dots \text{eq. 4}$$

Now, one casein = 24,000 Da (average molecular weight)

$$= 24000 \times 1.67 \times 10^{-27} \text{ kg (we know, 1 Da} = 1.67 \times 10^{-27} \text{ kg) .eq. 5}$$

Therefore, the number of caseins in that mass = total mass of micelle / mass of casein

$$\text{Number of caseins in the micelle} = (9.67 \times 10^{-19}) / (24000 \times 1.67 \times 10^{-27})$$

**Number of caseins in the micelle = 24126 .....eq. 6**

Now, the length of the total caseins = number of caseins x length of each casein

Roughly, the length of each casein = 20 nm

Therefore, total length = 24126 x 20 nm = 482520 nm  
.....eq. 7

Now, the big cube is made up of  $n$  small cubes having 12 ' $a_s$ ' sides:

So, the total length =  $n \times 12 \times a_s/4$  (divide by 4 because each cube shares with four other cubes)..... eq. 8

Also, total volume:  $a^3 = n * a_s^3$

$n = a^3/a_s^3$  ..... eq. 9

From eq. 7, eq. 8 and eq. 9:

total length = 482520 =  $n \times 12 \times a_s/4$  (where  $n = a^3/a_s^3$  is from eq. 9)

$= 482520 = \frac{a^3}{a_s^3} \times 12 \times a_s/4$

$= a_s = \sqrt[3]{4188790.25 * 3/482520}$

Therefore  **$a_s = 5.1$  nm**

If we consider that each small cube shares its sides with six cubes,

then  **$a_s = 4.16$  nm**

## Appendix 8. Conference poster presentations

Poster 1: Presented at 7<sup>th</sup> International Whey Conference, Rotterdam, Netherlands.

### Interactions of Whey Protein with Casein Play a Major Role in Preventing Plasmin-induced Hydrolysis in Heated Milk System

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INTRODUCTION

Sterilisation and UHT treatment are commonly used to increase the shelf life of milk and dairy beverages. However, the high heat resistance of the plasmin system enables it to partially survive UHT treatment, which could lead to defects in the texture (gelation, sedimentation) and the taste (bitterness) of these products.

The present study investigated the effect of the association of whey proteins with the casein micelles on the plasmin-induced hydrolysis of caseins.

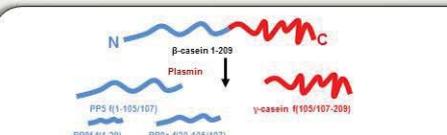


Fig. 1. Plasmin-induced hydrolysis of  $\beta$ -casein leads to the generation of  $\gamma$ -casein and proteose peptones, e.g. PPS, PPSs and PPSf.

EXPERIMENTAL PLAN

Lactose-free milk was prepared using microfiltration so that the intact state of the casein micelles was preserved. The milk system was high heat treated at 120°C for different times to achieve a range of association of the whey proteins with the casein micelles. All treated milk systems were then hydrolysed by adding plasmin and the hydrolysis reaction was monitored using reverse-phase high performance liquid chromatography (RP-HPLC). The extents of whey protein denaturation, dissociation of casein from the micelles and association of whey proteins with the micelles were monitored using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Particle size was monitored using dynamic light scattering.

**Milk System** → Lactose-free milk (pH 6.6) using microfiltration

**High Heat Treatment** → Milk system heated at 120°C for different times followed by immediate cooling

**Hydrolysis** → Plasmin: 0.1 U/ml for 0–8 h (37°C)

**Inactivation** → Inactivation by SDS

**Analysis** → Whey protein denaturation and association, Particle size, Hydrolysis pattern

RESULTS

► Upon high heat treatment (120°C), whey protein denatured rapidly, with the major effect on  $\beta$ -lactoglobulin compared with  $\alpha$ -lactalbumin (Fig. 2).

► The association of whey proteins with the casein micelles was slower, more progressive and more linear over time compared with whey protein denaturation (Fig. 3).

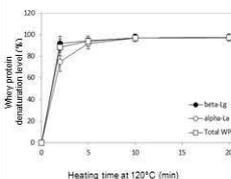


Fig. 2. The effect of high heat treatment on the level of whey protein denaturation (as measured by the decrease in pH-4.6-soluble whey proteins using RP-HPLC).

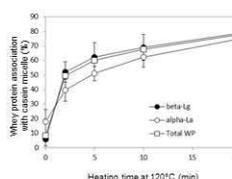


Fig. 3. The effect of high heat treatment on the association of whey proteins with the casein micelles (as measured by the decrease in the whey protein level in the serum phase of the milk using SDS-PAGE).

► The particle size increased continuously with an increase in the heat treatment and correlated with the association of whey proteins with the casein micelles (Fig. 3).

► The results from hydrolysis analysis using RP-HPLC (measurement of pH-4.6-soluble peptides) showed a linear decrease in plasmin-induced hydrolysis after the association of more than 40% of the whey proteins with the casein micelles. It could mainly have been due to whey protein covering the casein micelles and hindering the access of plasmin to different target caseins, i.e.  $\beta$ -casein and  $\alpha$ -casein.

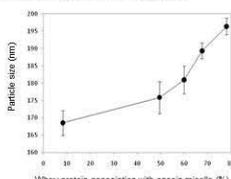


Fig. 4. The effect of high heat treatment on the change in particle size of the lactose-free milk system (as measured by dynamic light scattering).

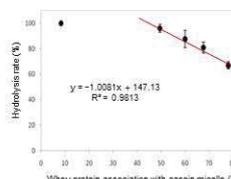


Fig. 5. The effect of the association of whey proteins with the casein micelles on the plasmin-induced hydrolysis rate (as measured by the generation of peptides using RP-HPLC).

PROPOSED MECHANISM

► Upon heat treatment, the association of whey proteins with  $\kappa$ -casein forms a covering around the micelle and thus limiting the access of plasmin to casein.

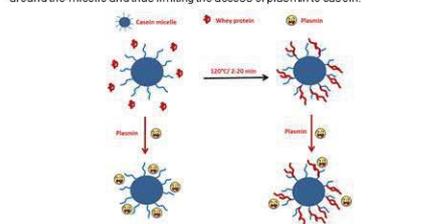


Fig. 6. Heat induced association of the whey protein with casein micelle limits the accessibility of plasmin to its targeted caseins, i.e.  $\alpha$ - $\beta$ -casein in the micelle.

CONCLUSIONS

► Plasmin-induced hydrolysis was found to decrease linearly with an increase in the association of whey proteins with the casein micelles.

► Whey protein denaturation did not correlate with the decrease in plasmin-induced hydrolysis. In contrast, particle size was well correlated because of its direct correlation with the association of whey proteins with the casein micelles.

► The decrease in plasmin-induced hydrolysis could be attributed mainly to whey proteins covering the micelles, thus limiting the access of plasmin to casein.

► The results and the relationship model from the present study will be very useful in optimising heat treatments to minimise plasmin-induced hydrolysis.

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Steve Taylor, Kerlene Higgs, Jean Gaman

Forever discovering



Te Kūmanga  
ki Pūrehuroa



# The mechanism of resistance to plasmin activity through protein succinylation: A model study using $\beta$ -casein

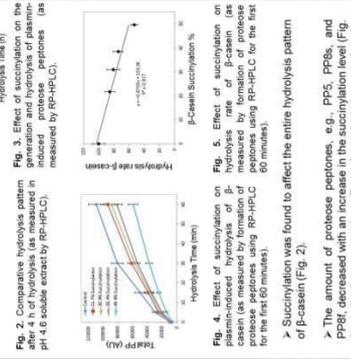
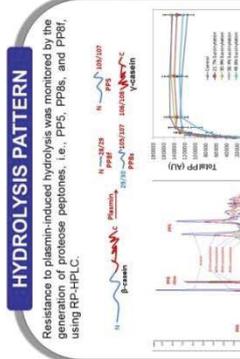
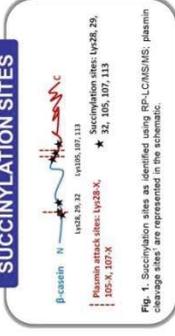
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**ABSTRACT**  
 Plasmin-induced proteolysis is a major concern in the dairy industry, leading to defects in the texture and taste, e.g., gelation and bitterness, of various UHT milk and beverages. It is therefore important to control plasmin activity in milk systems. Several techniques have been investigated, the present work explores inhibition of the plasmin-catalyzed hydrolysis of  $\beta$ -casein, which is the primary target of plasmin action.  
 As plasmin hydrolyzes proteins on the carboxyl site of lysine-X and arginine-X bonds, with a preference for the lysine-X bond, lysine residues on the  $\beta$ -casein backbone were targeted by succinylation. Succinylation sites were identified and the effect of succinylation on the plasmin-induced hydrolysis was presented by reversed phase liquid chromatography (RP-HPLC). The primary hydrolysis sites (RPLCMS) for the  $\beta$ -casein were Lys-29, Lys-105, Lys-107. To get greater insight into the effect of different levels of succinylation on the hydrolysis of  $\beta$ -casein by plasmin, the reaction was monitored by quantifying the formation of the hydrolyzed product using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and reverse-phase (RP-HPLC). This allowed the primary stage of the hydrolysis of  $\beta$ -casein, i.e., the formation of lysine-X and protease peptides, and the secondary stage, i.e., further hydrolysis of the lysine-X and protease peptides, to be distinguished.  
 The results clearly indicated that succinylation affected both stages of hydrolysis negatively. Succinylated  $\beta$ -casein and protease peptides became resistant to hydrolysis by plasmin. The formation of succinyl-lysine renders the casein unrecognizable to the substrate-binding pocket of plasmin. Thus, the modified substrate cannot be positioned into the pocket and is not hydrolyzed by the catalytic triad of plasmin. These results indicate that succinylation may be a potential mechanism for the inhibition of hydrolysis of milk proteins and that the proposed mechanism may be useful as a base hypothesis for developing plasmin-resistant proteins by other means of modification.

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**INTRODUCTION**  
 Bovine plasmin is a proteolytic enzyme that is present in low concentration in milk. Its activity on the major milk proteins, the caseins, leads to defects in the texture (gelation) and taste (bitterness) of dairy products. It is therefore important to control plasmin activity in dairy systems.  
 The present work investigated inhibition of the plasmin-catalyzed hydrolysis of  $\beta$ -casein, which is the primary target of plasmin. As plasmin hydrolyzes proteins on the carboxyl site of lysine-X and arginine-X bonds, with a preference for the lysine-X bond, lysine residues on the  $\beta$ -casein backbone were targeted by succinylation. Succinylation sites were identified and the effect of succinylation on the plasmin-induced hydrolysis with respect to the proposed mechanism is presented.

**EXPERIMENTAL PLAN**  
 Succinylation was achieved by adding succinic anhydride to the  $\beta$ -casein system. The succinylation level and the succinylated  $\beta$ -casein were identified using reverse-phase and RP-LCMS/MS methods. The effect of succinylation on the hydrolysis pattern by quantifying  $\gamma$ -casein (hydrolysis product) using SDS-PAGE and protease peptides using RP-HPLC.  
**Model System**  
 1.0%  $\beta$ -casein in 50 mM phosphate buffer (pH 6.7)  
**Succinylation**  
 Addition of succinic anhydride at different levels  $\rightarrow$  pH readjustment to 6.7  
**Hydrolysis**  
 Plasmin: 0.1 U/ml for 0-48 h (37°C)  $\rightarrow$  inactivation  
**Analysis**  
 Succinylation level: Site identification, Hydrolysis analysis



**HYDROLYSIS PATTERN**  
 Resistance to plasmin-induced hydrolysis was monitored by the generation of protease peptides, i.e., PP5, PP8, and PP9, using RP-HPLC.  
**PROPOSED MECHANISM**  
 Succinylation causes attachment of the succinate molecule to the  $\epsilon$ -amino group of the lysine residue and leads to charge modification and a change in steric availability, making the lysine residue unrecognizable to the substrate specificity pocket. Ultimately, the modified lysine residue leads to a reduction in hydrolysis or no hydrolysis (Fig. 6).  
**CONCLUSIONS**  
 > Lys-28, Lys-29, Lys-105, and Lys-107 are the primary target sites for succinylation.  
 > Succinylation makes protein resistant to plasmin-induced hydrolysis.  
 > The formation of succinyl-lysine alters the charge and steric availability, making the lysine residue unrecognizable to the substrate specificity and the binding to the specificity pocket of plasmin.  
 > The generation of PP5 and the further hydrolysis of PP5 are affected by succinylation.

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**ACKNOWLEDGEMENTS**  
 Steve Taylor, Katherine Higgs, Bettina Fong, Kees de Kruif



## Cross-linking of milk proteins can reduce its susceptibility to plasmin-induced hydrolysis

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### INTRODUCTION

Plasmin-induced proteolysis is a major concern in milk and dairy beverages, limiting their shelf life through the release of hydrolysed peptides. These peptides causing flavour (bitterness) and texture (gelation, sedimentation) defects<sup>1</sup>. Therefore it is important to control plasmin-induced proteolysis. The present work explores methods to control the plasmin-catalysed hydrolysis of  $\beta$ -casein (the primary target of plasmin), to minimise release of the defect-causing hydrolysed peptides.

As plasmin hydrolyses proteins on the carboxyl site of lysine-X and arginine-X bonds with a preference for the lysine-X bond<sup>2</sup>, lysine residues on the  $\beta$ -casein backbone were cross-linked with glutamyl residues to different extents. This was achieved by transglutamination, which is a transglutaminase (enzyme)-induced cross-linking reaction<sup>2</sup>.

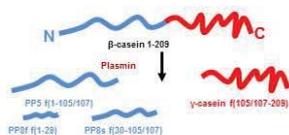
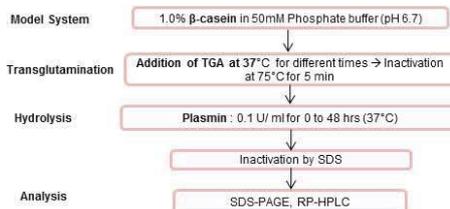


Fig 1: Plasmin-induced hydrolysis of  $\beta$ -casein leads to generation of  $\gamma$ -casein and proteose peptones e.g. PP5, PP6, PP8<sup>1</sup>.

### EXPERIMENTAL PLAN

Transglutamination was achieved by addition of TGA (transglutaminase) to  $\beta$ -casein model system at 37°C, followed by inactivation by heating at 75°C for 5 minutes. The resistance to hydrolysis was explored by adding plasmin at 37°C and analysing the hydrolysis pattern by quantifying one of the main product of hydrolysis proteose peptones using reversed phase-high performance liquid chromatography (RP-HPLC).



### HYDROLYSIS PATTERN: Proteose peptones using RP- HPLC

TGA-induced cross-linking was found to affect the entire hydrolysis pattern of  $\beta$ -casein (Fig. 2). There was a marked decrease in the hydrolysis when cross-linking was increased to more than 50% (Fig. 3).

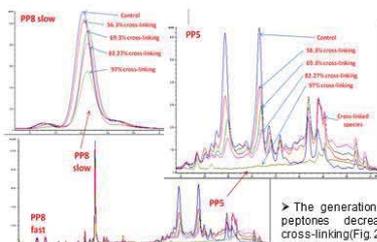


Fig. 2 Effect of TGA-induced cross-linking on plasmin-induced hydrolysis of  $\beta$ -casein (as measured by formation and hydrolysis of proteose peptones using RP-HPLC)

The results from RP-HPLC (Fig. 4) showed a linear decrease in the  $\beta$ -casein hydrolysis rate when cross-linking was increased to more than 50%, with an increase in the TGA-induced cross-linking.

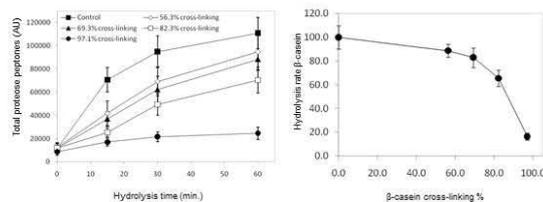


Fig. 3 Effect of TGA-induced cross-linking on generation of proteose peptones (as measured using RP-HPLC).

Fig. 4 Effect of TGA-induced cross-linking on hydrolysis rate of  $\beta$ -casein (as measured by generation of proteose peptones using RP-HPLC).

### PROPOSED MECHANISM

TGA-induced cross-linking involves glutamyl and lysine residues. The effect of such involvement of lysine residues in cross-linking has two implications: 1) modification of lysine into  $\epsilon$ -( $\gamma$ -glutamyl) lysine makes it unrecognisable to the substrate binding pocket of plasmin; therefore, the substrate is not positioned into the pocket and cannot be hydrolysed by the catalytic triad of plasmin. 2) Involvement of cross-linking will affect the release of hydrolysed peptides. The second effect appears to be playing the major role, as the effect became linear only after more than 50% of cross-linking.

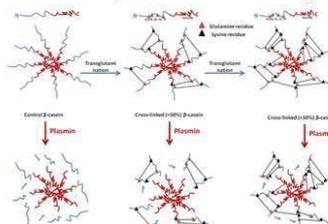


Fig. 5 Effect of transglutamination of  $\beta$ -casein on its hydrolysis by plasmin/plasmin.

### CONCLUSIONS

- The results clearly indicated that transglutamination affected plasmin-induced hydrolysis negatively.
- The involvement of lysine in cross-linking made it unrecognisable to the substrate binding pocket of plasmin and the cross-linking prevented the release of hydrolysed peptides.
- Thus transglutamination may be useful for controlling the plasmin-induced hydrolysis of milk proteins, to minimise the texture- and flavour-related defects that are caused by the release of hydrolysed peptides.

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Forever discovering



Te Kunenga  
 ki Pūrehuroa



# Plasmin resistance of the lactosylated protein

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## ABSTRACT

Bovine plasmin is present at low concentration in milk. However, high levels of plasmin in milk proteins, i.e. caseins may lead to defects in the texture and taste of some dairy products, e.g., proteolysis, gelation, and bitterness in UHT milk/beverages and protein products. It is therefore important to control plasmin activity in dairy systems. This work explores inhibition of the hydrolysis of  $\beta$ -casein catalyzed by plasmin.

The approach taken was to decrease  $\beta$ -casein availability by modifying it through processing. As plasmin hydrolyzes proteins on the carboxyl site of Lys-X and Arg-X bonds, with a preference for the Lys-X bond, lysine residues on the  $\beta$ -casein backbone were targeted. Substrate modification was achieved by lactosylation, which is the reaction between lactose and the amino group of lysine that leads to the formation of lactosyl-lysine.

To get a better insight into the effect of different levels of lactosylation on the hydrolysis of  $\beta$ -casein by plasmin, the reaction was monitored by quantifying the formation of the hydrolyzed product using sodium dodecyl sulfate polyanhydride gel electrophoresis and counter-phase liquid chromatography. The also characterized the different stages of the hydrolysis of  $\beta$ -casein that lead to the formation of  $\gamma$ -caseins and proteose peptones, and in the secondary stage to further hydrolysis of  $\gamma$ -caseins and proteose peptones.

The results clearly indicate the inhibitory effect of low levels of lactosylation on the hydrolysis of  $\beta$ -casein by plasmin. The mechanism of lactosyl-lysine renders the casein unrecognizable to the substrate-binding pocket of plasmin. Thus, the modified substrate cannot be positioned into the pocket and is not hydrolyzed by the catalytic triad of plasmin. These results indicate that lactosylation can be used to control the plasmin-induced hydrolysis of  $\beta$ -casein. The approach may be useful in preparing novel hydrolysates for the development of plasmin-resistant protein.

## ACKNOWLEDGEMENTS

Steve Taylor, Kerianne Higgs, Kees de Kraker, Stella Anema



## INTRODUCTION

Plasmin-induced proteolysis is a major concern in the dairy industry. Its activity leads to defects in the texture and taste, e.g., gelation and bitterness, of various milk systems. It is therefore important to control the activity of plasmin in milk systems.

This work investigated the inhibition of the plasmin-induced hydrolysis of  $\beta$ -casein. As plasmin hydrolyzes proteins on the carboxyl site of Lys-X and Arg-X bonds, with a preference for the Lys-X bond, lysine residues on the  $\beta$ -casein backbone were targeted (Fig. 1). The approach taken was to decrease the availability of the substrate by modifying the lysine group through lactosylation – the reaction between lactose and the  $\epsilon$ -amino group of lysine that leads to the formation of lactosyl-lysine<sup>1</sup>.

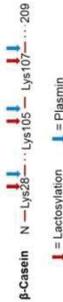


Fig. 1. Target sites of  $\beta$ -casein for lactosylation<sup>1</sup> and hydrolysis by plasmin<sup>1</sup>.

## EXPERIMENTAL PLAN

Lactosylation was achieved by heating  $\beta$ -casein and lactose under acidic conditions. The potential for hydrolysis was explored by adding plasmin at 37°C and analyzing the hydrolysis pattern by quantifying  $\gamma$ -casein (hydrolysis product) using SDS-PAGE and proteose peptones using RP-HPLC.

**Model System**  
 4.8% lactose and 1.0%  $\beta$ -casein in 50 mM phosphate buffer (pH 6.7)

**Lactosylation**  
 Heating of samples at 120°C/2, 5, 10, 20 min → immediate cooling

**Hydrolysis**  
 Plasmin: 0.1 U/ml for 0–48 h (37°C) → inactivation

**Analysis**  
 SDS-PAGE, UV spectrophotometer, Fluorescence spectrometer, RP-HPLC

## HYDROLYSIS PATTERN

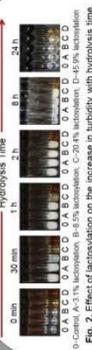


Fig. 2. Effect of lactosylation on the increase in turbidity with hydrolysis time. Control: A=0% lactosylation, B=5% lactosylation, C=20% lactosylation, D=45% lactosylation.  
 The peptides that are released by hydrolysis increase turbidity.  
 Based on the control, as the level of lactosylation increases, the increase in turbidity is delayed.  
 Visual observation (Fig. 2) indicated that lactosylation has a negative effect on plasmin-induced hydrolysis.

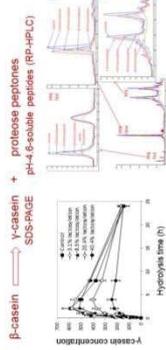


Fig. 3. Comparative hydrolysis pattern of  $\beta$ -casein and lactosylated  $\beta$ -casein using SDS-PAGE and proteose peptones using RP-HPLC.

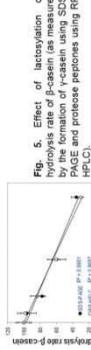


Fig. 4. Effect of lactosylation on the amount of proteose peptones released by the formation of  $\gamma$ -casein using SDS-PAGE and proteose peptones using RP-HPLC.  
 Lactosylation was found to affect the entire hydrolysis pattern of  $\beta$ -casein (Fig. 3). The higher the level of lactosylation, the lower the amount of  $\gamma$ -casein and proteose peptones released. A high lactosylation level (40.4%) became resistant to plasmin-induced hydrolysis.

The amount of proteose peptones, i.e., PP5, PP8, and PP9, decreased with an increase in the lactosylation level (Fig. 4), indicating a negative effect of lactosylation on plasmin-induced hydrolysis.  
 The  $\gamma$ -casein concentration decreased with lactosylation, indicating a decrease in the maximum amount of substrate being hydrolyzed (Fig. 3).

The results from SDS-PAGE (Fig. 3) and RP-HPLC (Fig. 4) showed identical linear decreases in the  $\beta$ -casein hydrolysis rate with an increase in the lactosylation level (Fig. 5).

## PROPOSED MECHANISM

Lactosylation causes attachment of the lactose molecule to the amino group of the lysine residue, making it unrecognizable to plasmin<sup>1</sup>

At higher lactosylation levels, cross-linking also affects the release of hydrolyzed peptides.

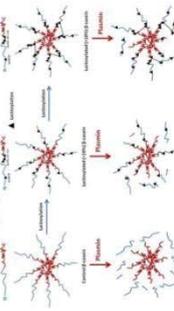


Fig. 6. Resistance of lactosylated  $\beta$ -casein to plasmin-induced hydrolysis. Lactosylation makes protein unrecognizable to plasmin. At higher lactosylation levels, cross-linking also affects the release of hydrolyzed peptides.

## CONCLUSIONS

- Lactosylation decreases plasmin-induced hydrolysis.
- The formation of lactosyl-lysine alters the charge and steric availability of lysine and this affects the substrate specificity and the binding to the specificity pocket of plasmin.
- At higher lactosylation levels, cross-linking (through the Maillard reaction) affects the release of the hydrolyzed peptides.
- The  $\beta$ -casein hydrolysis rate decreases linearly with an increase in the lactosylation level in the range tested.

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# Effect of micellar structure of casein and their modification on plasmin-induced hydrolysis

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## ABSTRACT

Plasmin-induced hydrolysis of casein in milk can lead to undesirable products, such as age related bitterness. We hypothesized that the susceptibility of casein micelles to plasmin can be affected by its micellar structure and modification of the lysine residues on caseins.

Different levels of dissociation of the casein micelle were achieved by the competitive chelation using EDTA and by succinylation by attaching a succinate group at the ε-amino group of lysine residues, leading to the formation of succinyl-lysine. The target sites for succinylation were identified using liquid chromatography-tandem mass spectrometry. Changes in the particle size and levels of dissociation were monitored using dynamic light scattering. Zeta potential and sodium dodecyl sulfate polyacrylamide gel electrophoresis, respectively. The subsequent plasmin hydrolysis was monitored by quantifying the hydrolyzed product using reverse phase high performance liquid chromatography.

The EDTA-induced calcium chelation and dissociation of the casein micelle increased plasmin-induced hydrolysis. In contrast, succinylation had an inhibitory effect on plasmin-induced hydrolysis. Calcium chelation and succinylation of the skim milk resulted in a decrease in turbidity, extensive unfolding, and changes collectively reduced steric hindrance and made the protein more readily hydrolyzed by plasmin. However, the formation of succinyl-lysine rendered β-casein unrecognizable to the substrate-binding pocket of plasmin, resulting in a nonlinear decrease in the hydrolysis because of the competitive effect. These results indicate that the competitive chelation indicate the importance of the casein micelle structure and its susceptibility to plasmin action. They will be useful in understanding and controlling the plasmin-induced hydrolysis of milk proteins in food systems.

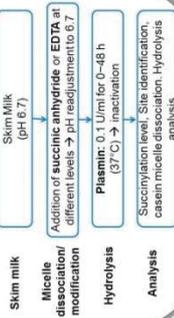
## INTRODUCTION

Bovine plasmin is a proteolytic enzyme that is present in low concentration in milk. Its activity on the major milk proteins, the caseins, leads to defects in the texture (gelation) and taste (bitterness) of UHT milk and beverages. It is therefore important to understand plasmin activity in dairy systems.

The present work investigated the susceptibility of casein micelles to plasmin affected by its micellar structure and modification of the lysine residues on caseins.

## EXPERIMENTAL PLAN

Micelle dissociation and modification was achieved by adding succinic anhydride or EDTA to the skim milk. The succinylation level was determined using RP-LCMS/MSMS. Resistance to hydrolysis was monitored by adding plasmin at 37°C and analyzing the hydrolysis pattern by quantifying using RP-HPLC.



## SUCCINYLACTION SITES

Plasmin sensitive lysine residues were targeted through competitive chelation to make these residues unrecognizable to plasmin.

Protein	Site (kDa)	Succinylation (%)	Yield (%)
α <sub>1</sub> -casein	Lys7-34, 83, 124	Lys7-34, 36, 42, 83	14
	Lys124	Lys124	12
	Lys124, 152, 168, 234	Lys152, 168, 234	24
β-casein	Lys-99, 107	Lys-107, 73, 99	11
	Lys-100	Lys-75, 77, 83, 91, 100, 135, 138	15

Fig. 1. Succinylation sites as identified using RP-LCMS/MSMS; plasmin cleavage sites are represented in the schematic.

## MICELLE DISSOCIATION PATTERN

With increase in EDTA concentration and succinylation the dissociation of casein from the micelle increased leading to increase in the porosity of the micelle as observed by decrease in the mean count rate.



Fig. 2. Effect of A. EDTA addition and B. succinylation on dissociation of caseins from the micelle as identified using SDS-PAGE.

The slight increase in the particle size upon succinylation indicates swelling of the micelle due to increase in the negative charges on the surface. Dissociation of casein from the micelle and increase in porosity could facilitate access to the plasmin sensitive proteins and could increase the hydrolysis rate.

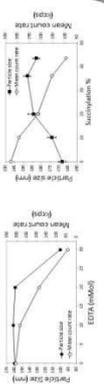


Fig. 3. Effect of A. EDTA addition and B. succinylation on particle size and mean count rate.

## HYDROLYSIS PATTERN

Plasmin-induced hydrolysis increased with an increase in micelle dissociation by EDTA addition (Fig. 4A).

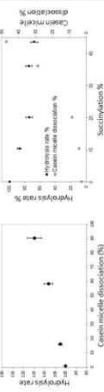


Fig. 4. Effect of A. EDTA addition and B. succinylation on plasmin-induced hydrolysis.

The hydrolysis rate was found to decrease linearly with increases in succinylation levels up to 20%. With further increases in succinylation levels the decrease in the rate became stagnant upto 36% and then there was a small decrease in the hydrolysis rate with further increases in succinylation to 43.60% (Fig. 4 B).

The halt in the further decrease of hydrolysis rate due to succinylation was in a correlation with the casein micelle dissociation (Fig 4B).

## PROPOSED MECHANISM

Plasmin-induced hydrolysis of casein is affected by casein micelle dissociation and casein modification. (1) Dissociation of caseins from the casein micelle due to succinylation or EDTA addition resulted in an increase in the turbidity and increase in micelle porosity, which collectively reduced steric hindrance and made the protein more readily hydrolyzed by plasmin. (2) Succinylation rendered caseins unrecognizable to the substrate-binding pocket of plasmin resulting in a decrease in the hydrolysis. The latter effect dominated the overall behavior and ultimately resulted in a decrease in the rate of hydrolysis with increases in the level of succinylation.

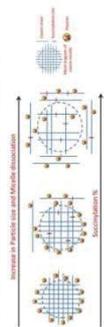


Fig. 5. Effect of succinylation of β-casein on its binding to the substrate-binding pocket of plasmin. The hydrolysis of bovine plasmin is based on its sequence homology with human plasmin and trypsin.

## CONCLUSIONS

- Protein modification at the lysine residues e.g. succinylation decreases the plasmin-induced hydrolysis and mechanism could be useful in making protein resistant through food grade modifications i.e. lactosylation or transglutamination.
- Casein micelle dissociation increases the protein accessibility to plasmin and ultimately increases the plasmin induced hydrolysis.
- Calcium chelation increases the plasmin-induced hydrolysis.

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## ACKNOWLEDGEMENTS

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# Elucidating the role of plasmin in sedimentation and age gelation in a heat-treated milk system

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## ABSTRACT

Sterilization and UHT treatment are commonly used in the dairy and food industries to increase the shelf-life of milk and dairy beverages. However, heat-resistant enzymes such as plasmin can partially survive UHT treatment, which may lead to defects in the texture and the taste of these products.  
 The present work aimed to study and provide more understanding of the role of plasmin in sedimentation and age gelation in heat treated milk, which is not yet fully understood.

The dissociation of peptides from the casein micelle, particle size, turbidity, changes in soluble minerals, and sedimentation were monitored. Two skim milk systems – (1) non-heat treated (NHT) and (2) heat treated (HT) (120°C/2 min) were compared in detail following the plasmin-induced hydrolysis to different extents. The dissociation of peptides from the casein micelle, particle size, turbidity, changes in soluble minerals, and sedimentation were monitored. The extent of hydrolysis, the size and composition of peptides were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis and reverse-phase high performance liquid chromatography (RP-HPLC).

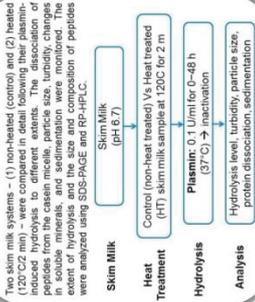
The hydrolysis patterns for the systems were markedly different. The heat-treated milk was associated with a greater proportion of peptides from the casein micelle of both hydrophobic peptides (e.g. γ-caseins) and hydrophilic peptides (e.g. protease peptones). The total calcium and phosphorus level in the serum samples were also higher in heat-treated samples than in control samples at the end of the hydrolysis. There was no sign of gelation in the whole system. Heat-treated samples had clear sedimentation whereas control samples showed sedimentation. It appeared that heat-induced association of whey proteins with the casein micelle followed by plasmin-induced hydrolysis of the casein micelle. These results provide further insights into the role of plasmin in sedimentation in heat-treated milk systems and improve our knowledge of why sedimentation occurs in heat-treated milk and beverage systems.

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## INTRODUCTION

Sterilization and UHT treatment are commonly used in the dairy and food industries to increase the shelf-life of milk and dairy products. However, heat-resistant enzymes such as plasmin can partially survive UHT treatment, which may lead to defects in the texture and the taste of these products.  
 The present work aimed to study and provide more understanding of the role of plasmin in sedimentation and age gelation in heat treated milk, which is not yet fully understood.

## EXPERIMENTAL PLAN



## SEDIMENTATION PATTERN

Sediment formation started only beyond 80% hydrolysis of β-casein.  
 The HT sample exhibited significantly more sedimentation than the control sample, which had hardly any visible sedimentation.  
 The sediment in the HT sample could mainly be attributed to the heat-induced association of β-lactoglobulin (β-LG) with κ-casein and the subsequent formation on the micelle surface.

Fig. 1. Effect of plasmin-induced sedimentation in heat treated (HT) and non-heat treated (NHT) samples.

## HYDROLYSIS-INDUCED CHANGES

With increasing levels of hydrolysis the turbidity (Fig. 2) and particle size (Fig. 3) of the milk samples decreased continuously in both HT and NHT samples up to around 80% β-casein hydrolysis and it can be explained by dissociation of the casein micelle upon plasmin-induced hydrolysis.

The dissociation of hydrolysed peptide, γ-casein and κ-casein increased gradually with increases in hydrolysis (Fig. 4A, B) and reached a maximum upon complete hydrolysis of β-casein.

Throughout the hydrolysis γ-casein and κ-casein dissociation pattern was higher in HT SM compared to NHT SM (Figure 4B).



Fig. 2. Effect of plasmin-induced hydrolysis on turbidity (as measured by absorbance at 700nm).



Fig. 3. Effect of plasmin-induced hydrolysis on particle size.

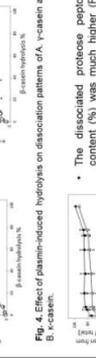


Fig. 4. Effect of plasmin-induced hydrolysis on dissociation patterns of A, γ-casein and B, κ-casein.

The dissociated protease peptone content (%) was much higher (Fig. 5) in the heat-treated milk from the start of the hydrolysis.

Throughout the hydrolysis time, the level of protease peptone dissociation was much higher in HT samples compared to NHT samples.



Fig. 5. Effect of plasmin-induced hydrolysis on dissociation patterns of protease peptones.

With increases in plasmin hydrolysis the total calcium and phosphorus level in the serum phase increased (Fig. 6), suggesting that the disintegration of casein micelle into CCP containing hydrophilic peptides or nanoclusters in agreement with the decrease in turbidity (Fig. 2), particle size (Fig. 3) and sedimentation (Fig. 1) of casein peptones in the serum phase (Fig. 5).

## CONCLUSIONS

- The present study explains plasmin-induced dissociation of the casein micelle in relation to physicochemical changes in both heat treated and non-heat treated milk systems.
- Upon plasmin-induced hydrolysis of the casein micelle, hydrophobic peptides, e.g. protease peptones, were the first to dissociate from the casein micelle, hydrophilic peptides, e.g. γ-caseins, dissociated slowly and with dissociation patterns that were identical to those of κ-casein, suggesting that release of κ-casein from the micelle was too slow to cause gelation.
- Extensive plasmin-induced hydrolysis of the casein micelle resulted in increased hydrolysis of κ-casein attributable to β-LG/κ-casein complex formation and its aggregation with hydrolysed hydrophobic peptides. These results provide new insights into the dissociation pattern of the casein micelle and how this relates to plasmin-induced sedimentation or gelation of UHT milk systems.

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Steve Taylor, Andrew Saunders, Kees de Kruif



## Plasmin Resistance of High-heat-treated Skim Milk: A Sequential Study

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### INTRODUCTION

Sterilisation and UHT treatment are commonly used to increase the shelf life of milk and dairy beverages. However, the high heat resistance of the plasmin system enables it to partially survive UHT treatment, which can lead to defects in the texture (gelation, sedimentation) and the taste (bitterness) of these products through plasmin-induced hydrolysis<sup>1</sup>.

A high-heat-treated (120°C/15 min) skim milk had been found to have higher resistance to plasmin-induced hydrolysis, compared with a control (unheated) skim milk. The exact reason and the mechanisms still need to be explained. The present study investigated the effect of high heat treatment on plasmin-induced hydrolysis using a sequential approach.

### RESULTS

The results of our study are presented in Figs. 1-3. It was observed that the particle size increased linearly with an increase in the intensity of heat treatment, which may be attributed to the association of whey proteins with the casein micelles (Fig. 1).

Moreover, the results presented in Fig. 2 clearly indicate that the lactosylation level increased with an increase in the holding time of samples at 120°C.

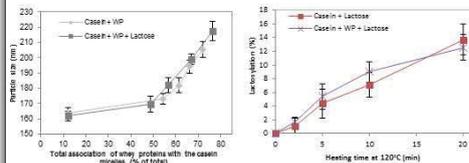


Fig. 1. The effect of high heat treatment on the change in particle size of the different milk systems (as measured by dynamic light scattering).

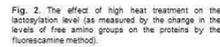


Fig. 2. The effect of high heat treatment on the lactosylation level (as measured by the change in the levels of free amino groups on the proteins by the fluorescamine method).

Plasmin-induced hydrolysis was found to decrease linearly in all except SPLFM samples, suggesting that whey proteins and lactose have a role in the action against plasmin (Fig. 3). When the results of the various treatments were compared, they indicated that SPFM showed the maximum decrease in the plasmin hydrolysis rate, followed by SM and LFM (Fig. 3).

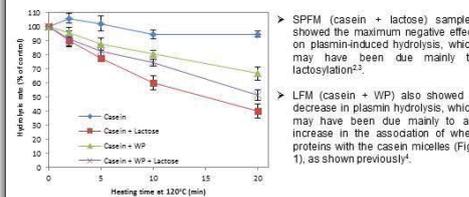


Fig. 3. The effect of high heat treatment on the plasmin-induced hydrolysis rate (as measured by the generation of peptides using RP-HPLC).

The linear decrease in plasmin-induced hydrolysis in SM (casein + lactose + WP) was due to both whey protein association and the lactosylation of caseins (Figs. 1 and 2).

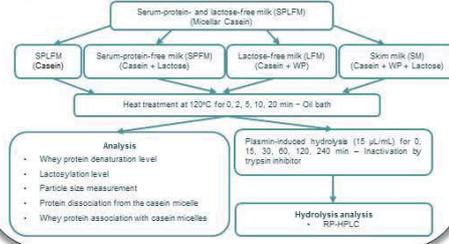
The negative effect on plasmin-induced hydrolysis was intermediate in SM (greater than in LFM but less than in SPFM) (Fig. 3), which could have been due to a distributive effect of lactosylation, wherein whey proteins are also lactosylated but are not the substrate for the action of plasmin.

High heat treatment of SPLFM (casein only) did not affect plasmin-induced hydrolysis, which could have been due to the absence of lactose and whey proteins.

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### EXPERIMENTAL PLAN

Different milk systems were prepared by removing whey protein and then lactose from skim milk and reconstituting each milk separately, to observe the effect of whey protein and lactose both individually and in combination. The milk systems were heated at 120°C for different times to achieve different levels of heat treatment. All treated milk systems were then hydrolysed by adding plasmin and the reaction was monitored using reverse-phase high performance liquid chromatography (RP-HPLC).



### PROPOSED MECHANISM

The plasmin resistance of high-heat-treated milk is explained by the schematic in Fig. 4. High heat treatment in the presence of lactose causes lactosylation (the formation of lactulosyl-His), making the protein unrecognisable to plasmin's specificity pocket<sup>2,3</sup> and resulting in a decrease in hydrolysis in SPFM (casein + lactose) and SM (casein + WP + lactose) samples.

On the heat treatment of lactose-free milk and skim milk systems (Fig. 4), whey proteins associate with the casein micelles (Fig. 1), such that the covering around the micelles limits the access of plasmin to casein<sup>4</sup>.

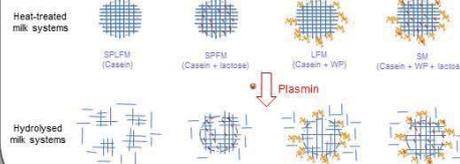


Fig. 4. A simplistic mesh diagrammatic representation of the mechanism for the plasmin resistance of high-heat-treated milk: mesh lines, casein chains; red lines, lactosylation sites; yellow lines, denatured whey proteins.

### CONCLUSIONS

Plasmin-induced hydrolysis was negatively affected by an increase in the heat treatment.

Both whey protein association with the casein micelles and lactosylation decreased the availability of protein to plasmin.

Whey-protein-free milk was the most resistant to plasmin, followed by skim milk and lactose-free milk, suggesting that lactosylation plays a more important role than whey protein association with the casein micelles in making protein resistant to plasmin-induced hydrolysis.

The results and the relationship model from the present study will be very useful in optimising heat treatments to minimise plasmin-induced hydrolysis.

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## Appendix 9. Statement of contribution

DRC 16



MASSEY UNIVERSITY  
GRADUATE RESEARCH SCHOOL

### STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Hemang Bhatt

Name/Title of Principal Supervisor: Rod Bennett

Name of Published Research Output and full reference:

Research Paper:

Bhatt, H., Cuchoval, A., Coker, C., Patel, H., Carr, A., & Bennett, R. (2014). Effect of lactosylation on plasmin-induced hydrolysis of  $\beta$ -casein. *International Dairy Journal*, 38 (2), 213 - 218. doi: 10.1016/j.idairyj.2014.01.017

In which Chapter is the Published Work: Chapter 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%  
and / or
- Describe the contribution that the candidate has made to the Published Work:

Hemang Bhatt  
Digital signature for Hemang Bhatt  
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IP: 134.110.100.100  
Candidate's Signature

08/12/2014  
Date

Rod Bennett  
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Principal Supervisor's signature

10/12/2014  
Date

GRC Version 3- 16 September 2011