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Structure modification of milk protein gels by enzymatic cross-linking

Dilek Ercili-Cura

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Abstract

Proteins are the structural building blocks of fermented, semi-solid dairy foods such as yoghurt. The nature of the protein-protein interactions and the structure of the macromolecular matrices they form determine the textural and water holding properties of a gel. In this study, enzymatic cross-linking of milk proteins in different colloidal and molecular states was studied. Effects of enzymatically formed inter-molecular covalent bonds on the gel formation dynamics and the structural properties of acid-induced milk protein gels were elucidated. The well-known protein cross-linking enzyme transglutaminase (TG), as well as the less studied oxidative enzymes *T. hirsuta* laccase, *A. bisporus* tyrosinase (AbT) and *T. reesei* tyrosinase (TrT) were used. Impacts of the cross-linkages formed by these enzymes, which differ from each other in their reaction mechanisms, were compared in the protein matrix of skim milk or caseinate gels.

Enzymatic protein cross-linking was mainly analysed by SDS-PAGE under reducing conditions. Laccase did not induce inter-molecular cross-links between casein molecules unless used in very high dosages. The use of ferulic acid (FA) as mediator in the laccase reactions increased the extent of cross-linking. In raw milk, TrT was the only enzyme that induced inter-molecular protein cross-linking. After heat-treatment of milk, both TG and TrT were able to form covalently linked oligomers. β-casein was the most readily cross-linked protein.

Susceptibility of the whey proteins to enzymatic modification is restricted due to their compact globular structure. After heat-treatment, which partially unfolds the whey protein molecules, both TG and TrT were capable of cross-linking whey proteins, whereas AbT and laccase were inefficient. In this respect, the extent of conformational change necessary for efficient cross-linking of β-lactoglobulin (BLG), the main whey protein, was studied by circular dichroism spectroscopy in various pH and heat-treatment conditions. At pH 7.5 and 9.0, although only a minor shift in the near-UV spectra of BLG was detected (only at pH 9.0), TG was able to induce cross-linking whereas TrT did not. After heat treatment (80 °C, 30 min), change in the ordered secondary structure and significant loss of the tertiary structure allowed both enzymes to form covalently linked BLG oligomers. Interestingly, the mobility on SDS-PAGE of the oligomer bands formed by TG was increased compared to the TrT-induced oligomers. In addition to different pH and heating conditions, adsorption to the air-water interface was also used as a means to change the molecular fold of BLG. BLG molecules adsorbed to the air-water interface were treated with TG, and possible inter-molecular cross-linking was indirectly analysed using interfacial shear rheology. TG action on the adsorbed molecules resulted in a decreased shear elastic modulus revealing that inter-molecular interactions were not enhanced but rather weakened by the enzyme treatment.

Small deformation oscillatory measurements and large deformation tests were utilized to characterize gel firmness of acid-induced milk or caseinate gels. Gel microstructures were visualized by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). Laccase, when used together with FA, increased the firmness of caseinate gels significantly. When raw milk was treated with TrT prior to acidification, the elastic modulus (G') of the final gel was found to be significantly higher compared to the control and the TG- or AbT-treated milk gels. In heated milk, however, TG was the only enzyme to increase the gel firmness as measured by G' and the large deformation measurements. Even though TrT did not affect these parameters, the tanδ value of TrT-treated sample was lowered compared to the control, revealing the introduction of new types of bonds into the protein matrix. Moreover, the peak in tanδ, which occurs after the gelation point during acidification of heated milk, was missing in both TG- and TrT-treated gels. The reason why TrT did not increase the gel firmness even though it showed substantial cross-linking was revealed by SEM imaging. Both the TG- and the TrT-treated heated milk gels were composed of considerably smaller gel particles compared to the control. The main difference between the TG- and the TrT-treated gels was observed to be in the inter-particle interactions. It appeared that intra-micellar cross-linking attained by both enzymes increased the stability of the casein micelles against dissociation upon removal of micellar calcium phosphate during acidification, while the reactivity of the micelles with each other was altered differently by the two enzymes. The results emphasized that intra-micellar cross-linking of casein micelles by both TG and TrT created altered gel particles and variable colloidal interactions.

Finally, the data of microstructural and textural characteristics of the acid milk gels produced from TG-treated skim milk were used to elucidate the mechanism behind improved water holding properties. Increased water holding capacity and prevention of whey separation has previously been reported for TG-treated acid milk gels. Together with rheology, gel formation was also followed with near-infrared light backscattering technique, and the microstructure and the water holding capacities of the control and the TG-treated heated milk gels were analysed during the course of the acidification. It was shown that at high acidification temperatures (>30 °C), the susceptibility of the protein network to large-scale rearrangements was significantly lowered when milk was pre-treated with TG at a high dosage. Smaller aggregate and pore sizes were observed in the gels from TG-treated milk compared to the untreated control gels throughout gel formation. Intra- and/or inter-particle covalent linkages introduced by TG were proposed to act as a fixative of the protein network at an early stage of gelation, thereby limiting the network contraction and subsequent whey separation.

Keywords milk, protein, tyrosinase, laccase, transglutaminase, cross-linking, acid gel

Preface

Research as a profession has been more than just a job for many and I am no exception. I feel very lucky to have the same passion with so many intelligent and inspirational people whom I worked with over the years at VTT, Technical Research Center of Finland. I am very grateful to Prof. Johanna Buchert and Prof. Kristiina Kruus for accepting me to VTT family as one of the Marie Curie fellows in the EU Project Pro-Enz (Enzymatic tailoring of polymer interactions in food matrix). Like many others, I always felt the freedom in my research, support throughout the entire process and constructive criticism. I would like to express my deep gratitude to Prof. Johanna Buchert. Your contagious enthusiasm and energy in science and positive attitude and care in life other than science have been always appreciated. Every PhD study has stories to tell but few have the highly motivational and cool supervisor in them. I was very fortunate to work under the supervision of Dr. Raija Lantto who gave me support and courage for going after my ideas but also did set me to the right track on those flighty moments, which were not few. Beginning is usually half the way but I think as researchers we know the true meaning of how to carry on. It makes life much easier when one can find true understanding and inspiration. I found my share in Dr. Riitta Partanen. I am very thankful to her for being by my side and pulling me up every time I got lost while asking too many "why" questions or when I was not able to deliver the last sentence in a manuscript. You have been a true mentor to me and I will appreciate this life-long. I sincerely thank to Martina Lille who introduced me the world of rheology and the rheometers after which we continued to explore together. I am very grateful to your endless support and good will and I believe we have made a nice and honest team in years.

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When you share your life with another researcher, you discover the true meaning of being supported, understood, and nurtured in this path to PhD. Simply, there are no words to express my gratitude to my husband Erkin. You have been everything to me, thank you…

Espoo, October 2012,
Dilek

Academic dissertation

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List of publications

This thesis is based on the following original publications, which are referred to in the text as I–IV (Appendix). The publications are reproduced with kind permission from the publishers

I Ercili-Cura, D., Partanen, R., Husband, F., Ridout, M., Macierzanka, A., Lille, M., Boer, H., Lantto, R., Buchert, J. & Mackie, A. R. 2012. Enzymatic cross-linking of β-lactoglobulin in solution and at air-water interface: Structural constraints. *Food Hydrocolloids, 28*, 1–9.

II Ercili Cura, D., Lantto, R., Lille, M., Andberg, M., Kruus, K., and Buchert, J. 2009. Laccase-aided protein modification: Effects on the structural properties of acidified sodium caseinate gels. International Dairy Journal, 19, 737–745.

III Ercili Cura, D., Lille, M., Partanen, R., Kruus, K., Buchert, J., and Lantto, R. 2010. Effect of *Trichoderma reesei* tyrosinase on rheology and microstructure of acidified milk gels. *International Dairy Journal, 20*, 830–837.

IV Ercili-Cura, D., Lille, M., Legland, D., Gaucel, S., Poutanen, K., Partanen, R., and Lantto, R. 2013. Structural mechanisms leading to improved water retention in acid milk gels by use of transglutaminase. *Food Hydrocolloids, 30*, 419–427.

Author's contribution to the appended publications

I The author planned the work together with Dr. Riitta Partanen. The author carried out the experimental work. Part of the experimental work was performed at IFR, Norwich, UK under the supervision of Dr. Alan Mackie. The author interpreted the data and had the main responsibility for writing of the publication, under the supervision of Dr. Riitta Partanen and Dr. Alan Mackie.

II The author planned the work together with Dr. Raija Lantto and Prof. Johanna Buchert. The author carried out the experimental work, interpreted the data and had the main responsibility for writing of the publication, under the supervision of Dr. Raija Lantto and Prof. Johanna Buchert.

III The author planned the work together with Dr. Raija Lantto and MSc. Martina Lille. The author carried out the experimental work (except the scanning electron microscopy which was performed by Unto Tapper), interpreted the data and had the main responsibility for writing of the publication, under the supervision of Dr. Raija Lantto and Dr. Riitta Partanen.

IV The author planned the work together with MSc. Martina Lille and Dr. Raija Lantto. The author carried out the experimental work. Image analysis was conducted by David Legland at INRA, France. The author interpreted the data and had the main responsibility for writing of the publication, under the supervision of Dr. Raija Lantto and Dr. Riitta Partanen.

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Publications I–IV

List of symbols

AbT	*Agaricus bisporus* tyrosinase
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ALA	α-lactalbumin
BLG	β-lactoglobulin
BS	Back scattering
BSA	Bovine serum albumin
CafA	Caffeic acid
CCP	Colloidal calcium phosphate
ChA	Chlorogenic acid
CLSM	Confocal Laser Scanning Microscopy
CMP	Caseinomacropeptide
cryo-TEM	cryo transmission electron microscope
DTT	1,4-dithiothreitol
FA	Ferulic acid
FESEM	Field emission scanning electron microscope
G''	Shear loss (viscous) modulus
G'	Shear storage (elastic) modulus
GDL	D-glucono-δ-lactone
GRAS	Generally regarded as safe
Lac	*Trametes hirsuta* laccase
L-DOPA	L-Dihydroxyphenylalanine
LVR	Linear viscoelastic region

MW	Molecular weight
nkat	Nanokatal
p-CA	*para*-coumaric acid
pI	Isoelectric point
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SEM	Scanning Electron Microscopy
$\tan\delta$	Loss tangent
TG	*Streptomyces mobaraensis* transglutaminase
TrT	*Trichoderma reesei* tyrosinase
WHC	Water holding capacity
WPI	Whey protein isolate

1. Introduction

Production of fermented milk products, such as yoghurt, relies on acid-induced gelation of milk proteins. Owing to their excellent functional properties, i.e., gelling, foaming, emulsifying and water holding, milk proteins constitute the most essential component of protein-stabilized dairy products. Their interactions and the structure of the three-dimensional protein network determine the textural and water retention (proneness to syneresis) properties of acid-induced milk gels.

Increasing interest in health-conscious or weight control-oriented eating habits necessitates novel and alternative ways to develop food products which promote and maintain satiety yet contain low energy-density (low-fat, reduced dry matter etc.). The challenge with low-calorie products is in maintaining the appealing sensory properties (texture and flavour) comparable to the originals. In the case of dairy products, especially yoghurt, low-fat or fat-free products often have poor texture, flavour and water holding properties, as fat globules are also essential structural elements. Fat globules partly fill the void areas in the protein matrix (Ciron et al., 2010; Le et al., 2011), giving the product a firmer texture (Xu et al., 2008) and they also contribute to perceived taste. Different methods have been studied and utilized in the dairy industry to improve the rheological and water holding properties of low-fat products. Some of these methods are listed in Table 1. Most of the methods, however, contribute to the energy content of the product and thus do not serve the aim completely.

The structural properties of gelled protein matrices are mainly determined by the number and strength of physical and chemical interactions (van der Waals attractions, hydrophobic, electrostatic interactions, hydrogen bonding, disulphide bridges) between protein molecules. One way of modulating protein interactions in such a system is the introduction of additional covalent bonds using protein cross-linking enzymes. Alteration of the functional properties of milk proteins through enzymatic cross-linking reactions may be exploited to manufacture dairy products with appealing texture despite low fat, reduced protein or reduced dry matter contents.

Table1. Potential processing aids for creating acceptable texture in non-fat or low-fat milk gels.

Method	Reference
Increasing the non-fat solids content	Lucey (2002); Sha et al. (2009)
Addition of thickening agents such as polysaccharides and gums	Saha & Bhattacharya (2010); Sanchez et al. (2000)
Imitating fat globules by addition of e.g. starch granules	Oh et al. (2007)
Natural formation of exopolysaccharides	Duboc & Mollet (2001)
Use of the protein cross-linking enzyme transglutaminase	Kuraishi et al. (2001); Buchert et al. (2007, 2010)

Transglutaminase (TG) is currently utilized for protein cross-linking in dairy, meat and baking applications. TG is commercially available and has had GRAS status since 1998. According to European Union (EU) legislations on food enzymes, transglutaminase is recognized as a processing aid (EU Regulation 1332/2008). In addition to transglutaminase, which is a transferase, oxidative enzymes such as tyrosinase and laccase have also shown the ability to induce covalent cross-links between proteins, with subsequent modification of the structural (Buchert et al., 2007, 2010; Jaros et al., 2006a; Kuraishi et al., 2001) and nutritional (Stanic et al., 2010; Tantoush et al., 2011; Monogioudi et al., 2011) properties of various food systems.

In order to tailor the protein functionality and the protein network structure towards improved mechanical properties of a final product, vast understanding of the molecular and colloidal interactions between various milk proteins is necessary. In the following literature survey, milk proteins and the formation and physical characteristics of acid-induced milk gels will first be reviewed. After that, enzymatic cross-linking of individual milk proteins and the subsequent effects on acid-induced milk gel structures will be elucidated.

1.1 Milk Proteins

Milk is both an oil-in-water emulsion and a dispersion of colloidal protein particles in a liquid phase which contains dissolved carbohydrates and salts. This colloidal nature and the influence of non-colloidal components (soluble proteins, lactose and minerals) on stability determine the processing behaviour and the final structure of the milk products.

Bovine milk contains approximately 3.5% (w/w) protein. The total nitrogen composition of bovine milk is distributed among caseins (~80%) and whey proteins (~15%), the remainder being non-protein nitrogen. Caseins constitute those proteins that can be precipitated at their isoelectric points (pI, ~pH 4.6) at temperatures >8 °C. They consist of four primary proteins, α_{s1}-, α_{s2}-, β-, and

κ-caseins, which show differences in their electrophoretic mobility, degree of phosphorylation, glycosylation and genetic polymorphism. The milk proteins that remain after the isoelectric precipitation of caseins are called whey proteins. They are comprised primarily of β-lactoglobulin (BLG) (50% of whey proteins) and α-lactalbumin (ALA) (20% of whey proteins), but also contain immunoglobulins, bovine serum albumin, lactoferrin and enzymes (lipases, proteinases, etc.) (Fox, 2003; Fox & Brodkorb, 2008; O'Regan et al., 2009). Some molecular characteristics of the main milk proteins are given in Table 2. Their molecular structures will be explained in more detail in the following sections.

Table 2. Some molecular characteristics of the main milk proteins (Fox, 2003; O'Regan et al., 2009; Swaisgood, 2003).

	MW (kDa)	Residues/molecule				
		Amino acids	**Phosphate**	**Pro**	**Cys**	**Intramolecular disulphide bonds**
Caseins						
α_{s1}-casein	23.6[a]	199	8[e]	17	0	0
α_{s2}-casein	25.2[b]	207	10–13	10	2	0
β-casein	24.0	209	5[f]	35	0	0
κ-casein	19.0[c,d]	169	1[g]	20	2	0
Whey proteins						
BLG	18.3	162	0	8	5	2
ALA	14	123	0	2	8	4

[a]Variant B, [b]Variant A, [c]Variant A^2, [d]Exclusive of carbohydrates [e]Occasionally 9, [f]Occasionally 4, [g]Occasionally 2 or 3

1.1.1 Casein micelles

Of the bovine caseins, α_{s1}, α_{s2}, and β caseins are highly phosphorylated on certain serine residues, which enables them bind Ca^{2+}. κ-casein, on the other hand, lacks the clusters of phosphoseryl residues, making it soluble in the presence of Ca ions. Moreover, κ-casein is glycosylated on the threonyl residues, which are all located in the C-terminal domain of the molecule. The glycosylated area of k-casein, called caseinomacropeptide (CMP), is the most hydrophilic part of the κ-casein molecule whereas the N-terminal domain (*para*-κ-casein) is very hydrophobic, thus giving the molecule an amphiphilic nature. Similarly, β-casein has a distinctly hydrophobic domain which comprises three fourths of the molecule including the C-terminal, and a highly charged N-terminal domain. The α_s-caseins also have hydrophobic patches along their sequence (Fox, 2003; O'Regan et al., 2009; Swaisgood, 2003). Caseins are known to have some ordered helical and β-structures, especially in their hydrophobic domains. Due to the conserved

features of their primary structure (high proline content), caseins do not attain a well-defined tertiary structure but maintain an open rheomorphic conformation (Holt & Sawyer, 1993). This rheomorphic structure gives caseins exceptional heat stability.

Considering the structural properties of caseins and the calcium phosphate content of milk, casein micelles can easily be depicted as association colloids which assemble mainly by hydrophobic interactions and calcium phosphate bridges. Whereas calcium-sensitive caseins form the interior structure, κ-caseins are located at the micelle surface with their hydrophilic CMP domain protruding out to the serum phase, forming a hairy layer on the micelle surface. This has been evidenced for example by the observed decrease of the micellar hydrodynamic diameter after renneting or ethanol treatment (De Kruif, 1999; Horne, 1986), and by microscopic analysis of the micelles (Dalgleish et al., 2004). Although caseins possess negative net charge at the physiological pH of milk, which prevents the proximity of the micelles to some extent, micelle-micelle interactions are mainly prevented by the steric stabilization enabled by the 'κ-casein hairy layer' (Dalgleish, 2011; Horne, 1986).

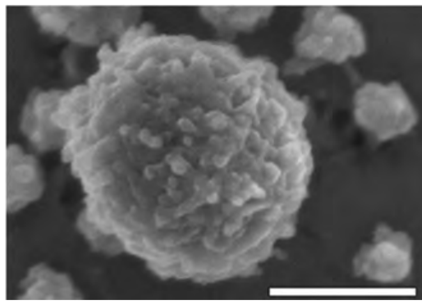

Figure 1. SEM image of a casein micelle. The bar represents 200 nm. Reprinted from (Dalgleish et al., 2004), copyright with permission from Elsevier.

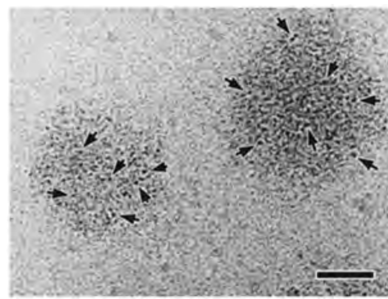

Figure 2. Cryo-TEM image of casein micelles. The bar represents 50 nm. Reprinted from (Marchin et al., 2007), copyright with permission from American Institute of Physics.

Casein micelles are highly hydrated, stable and polydisperse colloidal particles with a large size distribution of 50–500 nm. The diversity of the micellar size depends on the amount of κ-casein covering the micellar surface. κ-caseins can stabilize approximately 10 times their own mass of calcium-sensitive caseins (Fox, 2003). Various models for the micelle structure have been proposed (Table 3). Although there is a consensus between models about the surface location of the κ-caseins, the interior structure of the micelles, i.e. how the calcium-sensitive caseins are assembled and glued with colloidal calcium phosphate (CCP), is still under debate.

Recent advances in microscopical techniques have enabled detailed imaging of casein micelles and consequently better evaluation of the existing models. The field emission scanning electron microscope (FESEM) images (Figure 1, Dalgleish,

et al. 2004) have shown that the micelle surface is rough, with protruding tubular structures 10–20 nm in diameter and 40 nm in depth, indicating that the micelle structure might be more complex than a hard sphere covered with a hairy layer (Dalgleish et al., 2004). The interior of the micelles has been visualized by cryo transmission electron microscopy (cryo-TEM) (Knudsen & Skibsted, 2010; Marchin et al., 2007), which showed 2–3 nm-sized electron-dense regions evenly distributed inside the micelle, providing more evidence for calcium phosphate nanocluster models (Figure 2). The images also clearly show that the micellar surface contains pores or entrances, proving that the micelle interior is readily accessible from outside. Moreover, the micelle models which assign a major role to hydrophobic interactions in maintaining the micellar integrity (nanocluster and the dual-binding models) naturally suggest that the micelle interior contains domains or channels of water due to local exclusions (Dalgleish, 2011). Dissociation and re-association of β-casein from and to the micelle without affecting the micellar integrity upon temperature change has been related to the porous character of the micelles. It also implies the possible penetration of other molecules, such as enzymes, to the micelle interior.

Table 3. Existing models for casein micelle structure.

Model (Reference)	Proposed mechanism	Later arguments
'Sub-miceller model' **(Schmidt, 1980; Walstra, 1990)**	• α_s- and β-caseins polymerize via hydrophobic interactions forming ~14 nm-diametered submicelles. • Submicelles are cemented to each other by CCP. • The submicelles rich in κ-casein are located at the outer layers of the micelle.	• Poor explanations of why two kinds of sub-micelles (κ-casein rich and poor) would form. • Location of the CCP was not clearly established (Dalgleish, 2011; Horne, 2006). • The cement role of CCP was not found to be realistic as both calcium and phosphate are involved in the casein phosphorylation which occurs post-translationally (Holt, 1992). • Was not supported by later electron microscopy studies (McMahon & McManus 1998; Trejo et al., 2011)
'Calcium phosphate nanocluster model' **(Holt, 1992; Holt et al., 1998)**	• Phosphoseryl clusters of α_s- and β-caseins interact with CCP forming nanoclusters with a core radius of 2.3 nm. • As α_s-caseins carry multiple phosphoseryl clusters, they connect the nanoclusters to each other forming a growing network with a uniform distribution of nanoclusters.	• No substantive role was given to κ-casein • Did not explain how the micelle size is controlled • The average distance between the nanoclusters was estimated to be ~ 18 nm, overruling the bridging by a single molecule (Holt et al., 2003). • Weak interactions (hydrophobic, weak electrostatic interactions, hydrogen and ion bonding) between the tails of α_s and β-caseins protruding from the nanoclusters were underestimated (Horne, 2006).
'The dual binding model' **(Horne, 1998; Horne, 2006)**	• αs- and β-caseins act as multi-functional polymers interacting with CCP on one site (forming nanoclusters) and interacting with each other on their hydrophobic sites. • κ-casein terminates the chain growth as it lacks the Ca-binding site, thus naturally locating on the surface of the micelle with its hydrophilic tail protruding.	• Describes the intra-micellar interactions but does define the interior structure of the micelle (Dalgleish, 2011). • The interaction of only a pair of molecules was suggested to be thermodynamically unfavourable (de Kruif et al., 2012). • Only monomeric κ-casein molecules were depicted (Farrel et al., 2006)

1.1.2 Whey proteins

Unlike the rheomorphic structure of caseins, whey proteins are compact globular proteins with well-defined secondary and tertiary structures. They are more heat sensitive and less calcium sensitive than caseins. The most abundant whey protein in bovine milk is β-lactoglobulin (BLG). It belongs to the lipocalyn family of proteins due to its ability to bind small hydrophobic molecules into its internal hydrophobic cavity (Papiz et al., 1986; Sawyer & Kontopidis, 2000). The monomeric diameter of the BLG molecule was reported to be ~3.5 nm (Sawyer & Kontopidis, 2000). Under physiological conditions, bovine BLG exists as a homodimer. The BLG monomer contains one free cysteine and two disulphide bridges. It is folded in a calyx-shaped β-barrel structure. The free cysteine remains buried inside the molecule in native state and becomes exposed and reactive only after a change in tertiary structure (Brownlow et al., 1997; Considine et al., 2007; Qin et al., 1998). The other major whey protein is α-lactalbumin (ALA). In its native form, ALA contains eight cysteine residues which are linked together by four disulphide bridges. It has an ellipsoid-like shape with dimensions 2–4 nm (Fox, 2003).

Heating of milk at temperatures >70 °C causes dissociation of the BLG dimer, partial unfolding and aggregation via hydrophobic associations, and thiol-disulphide exchange reactions (Considine et al., 2007). The reactive thiol of heat-denatured BLG can react with other whey proteins as well as κ-caseins on the surface of the micelles, forming micelle-bound and also soluble κ-casein/whey protein complexes in milk. The extent of such aggregate formation depends on the temperature of heating, which affects the whey protein denaturation, and the pH during heating which affects the distribution of bound and soluble complexes (Anema, 2009b; Anema & Li, 2003; Guyomarc'h et al., 2003; Vasbinder & de Kruif, 2003). Thus, in heated milk, the surface of the micelles is modified by coupling of whey protein aggregates and κ-caseins. In addition, milk serum contains soluble whey protein/κ-casein and pure whey protein aggregates. Guyomarc'h et al. (2003) estimated the soluble complexes to be either spherical particles of around 10 nm diameter or long fibrous particles several hundred nm in length.

1.2 Acid-induced milk gels

For yoghurt production, milk is acidified by starter bacteria which slowly ferment lactose to lactic acid, causing a gradual decrease in pH. For research purposes, however, direct addition of acids such as HCl, or addition of glucono-δ-lactone (GDL) is often used. The gradual pH-dropping effect obtained by GDL hydrolysis mimics bacterial fermentation, although the rate of acidification during the initial stages is different. GDL hydrolysis leads to a rapid initial pH drop, whereas with bacterial cultures a lag period is observed prior to the rapid pH drop. According to Lucey et al. (1998a), the difference in the rate of acidification at this critical initial stage causes some modifications in structural properties of GDL-acidified gels compared to gels acidified with bacteria.

1.2.1 Formation of a gel: physico-chemical interactions

Upon acidification of raw milk or caseinate solutions, proteins aggregate due to gradual loss of electrostatic repulsions, as well as other physical interactions such as hydrophobic and van der Waals attractions and steric and entropic effects (Roefs & van Vliet, 1990). In milk, the casein micelles undergo changes in their physico-chemical characteristics during pH drop which finally lead to micelle-micelle interactions and gradual formation of the three-dimensional protein network. The changes occurring consecutively are explained in the relevant pH ranges below:

6.7 > pH > 6.0
The net negative charge on the casein micelles decreases with decreasing pH, resulting in the reduction of electrostatic repulsions (Lucey, 2007; Phadungath, 2005). The casein micelles retain their size, shape and integrity. CCP solubilisation is very slow until pH 6.0–5.8 (Gastaldi et al., 1996).

6.0 > pH > 5.0
The negative net charge continues to decrease, resulting in the loss of electrostatic repulsion which also leads to collapse of the κ-casein hairy layer on the surface of the micelles. Thus, both the electrostatic and steric stabilization mechanisms of the micelles are gradually lost. CCP solubilisation increases after pH 5.8 and reaches its highest rate at around pH 5.1 (Gastaldi et al., 1996; Lucey, 2007; Phadungath, 2005). As CCP is largely responsible for the integrity of the casein micelles, its depletion causes dissociation of individual caseins, mainly β-caseins, from the micelles to some extent depending on both pH and temperature. Considerable casein dissociation was reported at acidification temperatures of 15 °C and 20 °C (Banon & Hardy, 1992). On the other hand, no casein dissociation was observed when acidification was performed at 30 °C or higher, implying the importance of hydrophobic interactions in the internal stability of the micelles (Banon & Hardy, 1992; Dalgleish & Law, 1988). Although both the internal and external properties of the casein micelles change considerably, only little change in the average hydrodynamic diameter has been observed above pH 5.0 (Banon & Hardy, 1992; Lucey & Singh, 1998). However, microscopical analysis (using SEM) showed that the newly formed calcium-depleted micelle particles became deformed around pH 5.3 and that coalescence of the particles started (Gastaldi et al., 1996).

5.0 > pH > 4.6
As acidification approaches the pI of caseins, casein-casein aggregation via electrostatic, hydrophobic and other weak interactions (van der Waals, H-bonding) intensifies. Increased ionic strength of the serum phase due to the CCP solubilisation also strengthens electrostatic interactions. In raw milk, gel formation starts at around pH 4.9 unless a high acidification temperature is used which would lead to the onset of gelation at higher pH values (Lucey, 2007; Phadungath, 2005). After

that point, fusion of the casein particles, organization of strands and clusters and further rearrangement of the three dimensional network continues.

When milk is subjected to high temperature (>70°C) heat treatments prior to acidification, the denaturation of whey proteins and subsequent formation of micelle-bound κ-casein/whey protein complexes result in modified surface properties of the micelles. Accordingly, both surface hydrophobicity and the apparent pI increase (towards the pI of whey proteins) (Donato & Guyomarc'h, 2009; Lucey et al., 1998b; Lucey et al., 1997). As a consequence, gel formation starts at higher pH values (~pH 5.4 depending on the pre-heating conditions and the acidification temperature) in heated milk compared to raw milk. Finally, in heated milk gels, whey proteins and disulphide bridges are incorporated into the gel network, whereas in unheated milk the gel network is mainly composed of weak casein-casein interactions.

Formation of caseinate gels, however, is a less complex phenomenon. At neutral pH, in solution at a comparable protein content to that of milk, sodium caseinate exists as self-assembled aggregates (with a radius of ~10 nm) of different caseins (Ruis et al., 2007) which gel upon decreasing pH towards their pI.

1.2.2 Rheological properties

Acid-induced casein or milk gels are considered to be particle gels, meaning that they are formed of aggregated spherical particles forming a continuous network of clusters and strands (Horne, 1999). However, they differ from gels formed of hard spheres as the internal structure of the building blocks, i.e. casein micelles greatly affect the gel properties (Horne, 2003; van Vliet et al., 2004). Accordingly, van Vliet et al. (2004) proposed that acid milk gels can be considered as particle gels during gel formation but soon afterwards they exhibit properties coherent with both particle and polymer gels. The polymer gel properties result from the fusion of casein particles during gel formation, after which strand deformation is not limited to the inter-particle regions.

A gel is a viscoelastic solid (Horne, 1999). The mechanical properties of an acid-induced milk gel during and after gel formation can be identified by small deformation oscillatory measurements (see section 2.5.1) and various large deformation tests (see section 2.5.2) applied to the gel.

Rheological properties of an acid-induced milk gel are associated with the number and type (strength) of the bonds between and inside the casein particles and their spatial distribution, meaning the curvature and thickness of the strands making up the gel network (Lakemond & van Vliet, 2008b; Mellema et al., 2002a; Mellema et al., 2002b; Roefs et al., 1990; Roefs & Van Vliet, 1990; van Vliet et al., 2004). In dynamic measurements, the evolution of the moduli (G', G'', or G*) is followed during gel formation. First a lag period is observed until the gelation pH is reached and after that point, a sudden increase followed by a plateau is common. Meanwhile, loss tangent ($\tan\delta = G''/G'$) makes a sharp decrease and reaches 1 ($G'' = G'$) at the gelation pH. It then continues to decrease, first sharply then gradually until a value of ~0.25 is reached for acid milk gels (Lucey & Singh, 1998). In

large deformation measurements, the fracture stress (force) and/or strain is measured. Accordingly, the strands making up the gel network are straightened, stretched and finally broken.

Acid gels made from heat treated milk show markedly higher final G' values compared to raw milk gels. In heated milk gels, increased micellar surface hydrophobicity and introduction of disulphide bridges (which is lacking in raw milk gels) contribute to the strength of connections between the particles, leading to enhanced mechanical properties (Lucey et al., 1998b; Lucey et al., 1997; Vasbinder & de Kruif, 2003). The role of soluble and micelle-bound whey protein/ κ-casein complexes in structure formation and the properties of acid-induced milk gels have been extensively reviewed (Donato & Guyomarc'h, 2009; Morand et al., 2011). Another significant difference observed between raw and heated milk gels is the occurrence of a local maximum in $\tan\delta$ immediately after the gelation point in heated milk. It is mainly related to the partial loosening of the intra-micellar bonds due to removal of micellar calcium phosphate (Anema, 2008, 2009a; Horne, 2003; Lakemond & van Vliet, 2008a; Lucey, 2002; Lucey et al., 1998b). The temporary loosening of intra-micellar interactions leads to increasing $\tan\delta$ values, which then start to decrease due to the enhanced electrostatic interactions creating a local maximum. At elevated (>40°C) acidification temperatures, it is also reflected in G' by causing a shoulder simultaneously with the $\tan\delta$ peak (Anema, 2009a). The reason why such a peak in $\tan\delta$ does not occur in raw milk is the low gelation pH in raw milk; CCP solubilisation is already completed before the gelation point is reached. The final value of $\tan\delta$ is also a good measure of the dynamic and viscoelastic character of acid gels. In fact, Lakemond & van Vliet (2008) indicated that change in $\tan\delta$ is a more direct indication of a change in type and strength of interactions in a gel network compared to the G'.

1.2.3 Microstructure and water holding properties

Acid-induced skim milk (or caseinate) gels are visualised as protein particles linked together in clusters and strands forming a coarse network (Kalab et al., 1983). The structure is shown in high magnification in Figure 3. Water (or whey) is physically entrapped in the pores of the gel network. The size of the pores, i.e. the coarseness of the structure depends greatly on protein concentration, casein to whey protein ratio (if changed), pre-heating conditions and aggregation dynamics, which in turn depend on temperature and the rate of acidification.

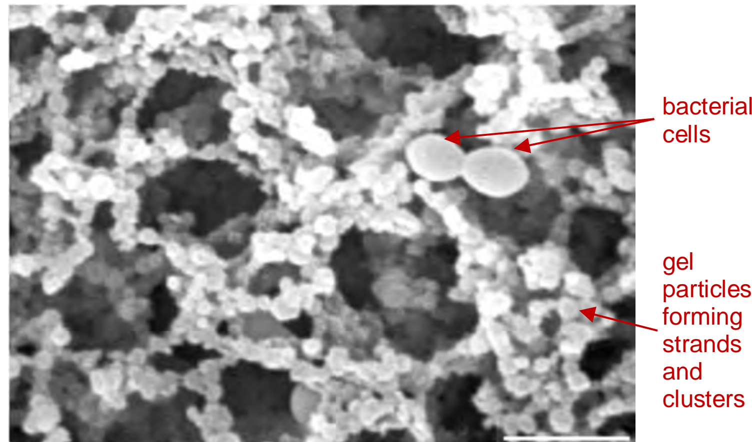

Figure 3. SEM image of yoghurt fermented with lactic acid bacteria. Casein particles form clusters and strands entrapping the water phase in the pores. Two bacterial cells are also visible. The scale bar represents 2 μm. Modified from Kalab (2010).

Heat treatment of milk prior to acidification results in a different microstructure compared to raw milk gels. Gels from unheated milk show aggregates or clusters of protein particles unevenly distributed in the gel matrix and with less connectivity in between. On the other hand, acid gels from heat-treated milk are described as 'branched', with more visible interconnectivity between the aggregates, and show more homogeneous network structure compared to raw milk gels (Lucey et al., 1998b, 2001, 1998c).

The water content of a skim milk gel (without any dry matter fortification) is typically around 90%. Retention of water in the gel structure is an important functional property and is directly related to the network structure (Hermansson, 2008; van Vliet & Walstra, 1994). Spontaneous syneresis (or whey separation), which refers to separation of water (or whey) from the gel without any applied external force, is a common defect in acid-induced milk gels. Water in such gels is physically entrapped within the casein strands forming the gel network, meaning that the tendency for whey separation is primarily linked to the dynamics of the network (van Vliet & Walstra, 1994). Accordingly, proneness of the casein network to large-scale rearrangements during and after gel formation is the most important factor determining water retention in such gels (Walstra et al., 1985). Continuous rearrangements lead to contraction of the gel, causing inability to entrap all the water. The higher rearrangement potential of a casein particle network can be inferred for example from a high tanδ value. Accordingly, a high tanδ during the initial phases of gel formation or high final tanδ would indicate that relatively more protein-protein bonds relax per cycle of oscillation, implying faster yielding of the interactions and thus proneness of the network to further rearrangements (van Vliet & Walstra, 1994; van Vliet et al., 1991). High acidification temperature, fast acidification rate and excessive pre-heat treatment (when conjugated with high

acidification temperatures) have all been identified to lead to increased whey separation in milk gels (Lucey, 2002).

1.3 Enzymatic cross-linking of milk proteins

In the food industry, enzymes have long been used as processing aids for improving textural, sensorial, or nutritional attributes in a green and economical way. However, protein cross-linking enzymes have gained interest only during the past few decades, mainly since the production of Ca^{2+} independent microbial TG in 1989 (Ando et al., 1989). TG has been widely studied and applied in meat products (as reviewed by Marques et al., 2010) but also in dairy (as reviewed by Jaros, et al. (2006a)) and cereal (Autio et al., 2005; Steffolani et al., 2008; Takacs et al., 2008) applications. In addition to TG, oxidative enzymes; laccases, tyrosinases, peroxidases and sulfhydryl oxidases, have shown potential to form covalent links in food protein substrates (Buchert et al., 2010). Among these, the reaction mechanisms of transglutaminase, laccase and tyrosinase will be briefly described in the following section. Later, recent literature on cross-linking of caseins and whey proteins as affected by their molecular and colloidal states will be summarized.

1.3.1 Cross-linking enzymes: Transglutaminase, laccase and tyrosinase

TG (glutaminylpeptide:amine γ-glutamyltransferase, EC 2.3.2.13) catalyzes an acyl transfer reaction between the γ-carboxyamide group of a protein-bound glutamine residue and a primary amine or the amino group of a protein-bound lysine residue, leading to (γ-glutamyl)-lysine isopeptide linkages (Folk & Finlayson, 1977; Griffin et al., 2002) (Table 4). In the absence of amines, water serves as acyl acceptor leading to the conversion of glutamines to glutamic acid (deamidation) (Griffin et al., 2002; Kashiwagi et al., 2002). Commercially available microbial TG from *S. mobaraensis* is a monomeric protein with a MW of 38 kDa. It shows optimum activity in the pH range 5–8 (Ando et al., 1989).

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are copper-containing oxidases which generate free radicals and utilize molecular oxygen as an electron acceptor. The physiological functions of laccases are mostly related to polymerization or degradation reactions of lignin (Gianfreda et al., 1999; Thurston, 1994). They can also act on certain amino acid residues in proteins. Laccase-catalyzed oligomerization of peptides or proteins proceeds through oxidation, mainly of tyrosine (Mattinen et al., 2005, 2006), and possibly also cysteine residues (Figueroa-Espinoza et al., 1998; Labat et al., 2000). The mechanism of laccase-catalyzed oxidation of tyrosine-containing peptides has been proposed to proceed via generation of radicals in the hydroxyl group of the phenolic ring, with concomitant generation of a semiquinone and rapid delocalization of the radical into the different positions of the aromatic ring (Mattinen et al., 2005) (Table 4). Mattinen et al. (2005) have also shown that iso-dityrosine bonds are formed when hydroxyl and tyrosyl radicals located in different molecules react with each other.

Relatively poor reactivity of proteins with laccase has been assumed to be due to the limited accessibility of the tyrosine residues in proteins (Mattinen et al., 2006), or alternatively due to their high redox potential (Xu, 1996). Thus the presence of a mediator or auxiliary substance is most often needed. Mediators are small molecules that are readily oxidized by laccase, producing radicals which can then react with the target substrate. Laccases show wide variation in their MW and optimum activity conditions depending on their origin. For phenolic substrates, the optimum pH can vary between 3 and 7 for fungal laccases (Xu, 1999). They exist mainly as monomers or homodimers with a MW range of 60–100 kDa, and have a low degree of glycosylation (Solomon et al., 1996; Xu, 1999).

Tyrosinases (monophenol, *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) are multicopper oxygenases which are widely distributed in nature, and they are mainly involved in the biosynthesis of melanin pigments (Claus and Decker, 2006). Tyrosinases catalyse two distinct reactions; *ortho*-hydroxylation of monophenols (monophenolase or cresolase activity) and oxidation of diphenols (diphenolase or catecholase activity) with subsequent formation of *ortho*-quinones (Table 4). Oxygen is the co-substrate in both reactions (Solomon et al., 1996). In addition to their natural substrates tyrosine and dihydroxyphenylalanine (DOPA), tyrosinases are capable of oxidizing various mono- or diphenols having a similar structure to these compounds. In protein systems, tyrosinase is known to induce covalent cross-linking by oxidizing tyrosine residues to the corresponding quinones, which further react non-enzymatically with each other or with free sulphydryl and amino groups resulting in the formation of tyrosine-tyrosine, tyrosine-cysteine and tyrosine-lysine cross-links (Burzio et al., 2000; Ito et al., 1984; Matheis & Whitaker, 1984; Takasaki & Kawakishi, 1997). Recently, a novel cross-link between tyrosine and histidine side chains has also been reported upon tyrosinase treatment of a model protein (Hellman et al., 2011). The MW of well-characterised bacterial and fungal tyrosinases are reported to be 40–50 kDa (Solomon et al., 1996). Fungal tyrosinases show optimum activity mainly at neutral or slightly acidic pH (Buchert et al., 2010), whereas some fungal tyrosinases, e.g. tyrosinase isolated from *T. reesei* (Selinheimo et al., 2006) show optimum activity at alkaline pH.

Depending on their origins (fungal species, bacterial, plant etc.), each enzyme class shows internal differences in their ability to oxidize proteinecious substrates and to form cross-links.

Table 4. Catalytic activity of transglutaminase (TG), laccase and tyrosinase on protein-bound reactive residues.

Enzyme	Primary reaction	Example of cross-linking
TG[a]	Gln — C(=O) — NH_2 + H_2N — Lys	Protein — $(CH_2)_2$ — C(=O) — NH — $(CH_2)_4$ — Protein
Laccase[b,c]	4 OH, R ⇌ (- 4H+) ⇌ (- 4e-) [•O, R ⇌ O, R ⇌ O, R ⇌ O, R] 4	OH, Protein, O, Protein
Tyrosinase[d]	R, OH → (Monophenolase, 1/2 O_2) → R, HO, OH → (Diphenolase, 1/2 O_2, H_2O) → R, O, O	Protein, HO, OH, OH, OH, Protein

[a]modified from (Kuraishi et al., 2001), [b]modified from (Mattinen et al., 2005), [c]Validated for high redox-potential laccases, [d]modified from (Claus & Decker, 2006) and (Selinheimo et al., 2008).

1.3.2 Impact of protein structure and colloidal state on enzymatic cross-linking

The formation and extent of enzyme-induced inter- and/or intra-molecular cross-links in protein systems can be related to factors such as use of optimum activity and stability conditions (temperature, pH, exclusion of inhibitors, etc.) for the enzyme, and the morphological state of the substrate protein molecule in the reaction conditions, i.e. the size and shape of the molecule and accessibility of the target amino acid side-chains (Hellman et al., 2011; Mattinen et al., 2008a; Mattinen et al., 2006; Partanen et al., 2011). Moreover, proximity of the target molecules participating in inter-molecular cross-linking is crucial and is affected by the concentration and physiochemical properties (hydrophobicity, ζ-potential).

Proteins without a confined tertiary structure are more prone to enzymatic cross-linking compared to highly compact globular proteins in which target amino acid residues can be embedded in the interior of the molecule and thus inaccessible. In this respect, caseins are good substrates for enzymatic cross-linking because of their flexible rheomorphic structure, whereas globular whey proteins in their native form are poor substrates for any type of enzymatic catalysis. Complete or partial denaturation of the whey proteins by means of chemical reduction of the disulphide bridges or by exposure to alkaline pH, high temperature, or high pressure treatments can however increase the extent of enzymatic cross-linking of these proteins (see reviews by Buchert et al., 2010; Faergemand et al., 1998; Huppertz, 2009; Jaros et al., 2006a; Thalmann & Lötzbeyer, 2002). Some recent studies on the cross-linking of individual milk proteins are summarized in Table 5 for caseins and Table 6 for whey proteins.

Another condition that can affect the susceptibility of globular proteins to enzymatic reactions could be the adsorption to interfaces. Proteins are surface active in nature and it has long been hypothesized that once they adsorb to interfaces, they partially unfold and arrange their conformation such that hydrophobic sites align with the hydrophobic phase (see reviews by Bos & van Vliet, 2001; Murray, 2002; Wilde, 2000), which is limited in a tightly packed monolayer (Dickinson, 1997). Conformational studies on adsorbed layers of BLG have shown that the change in secondary structure is highly limited irrespective of the surface concentration (Lad et al., 2006; Martin et al., 2003; Meinders & de Jongh, 2002), and no decisive conclusions have yet been reported on tertiary structure (Wierenga & Gruppen, 2010). However, formation of inter-molecular disulphide bonds in adsorbed BLG layers was proposed (Dickinson & Matsumura, 1991), which would require a certain change in tertiary structure. Similarly, BLG was reported to be more effectively cross-linked by TG when it was adsorbed on the surface than in bulk (Færgemand et al., 1997), and cross-linking was more pronounced at oil/water interface compared to air-water interface due to greater unfolding at oil/water interface (Færgemand & Murray, 1998).

Owing to the colloidal stability of casein micelles and the high casein concentration inside the micelle, mainly intra-micellar cross-linking takes place in milk. This is evidenced by; lack of significant particle size difference in milk even

though caseins are substantially cross-linked with transglutaminase (Huppertz & de Kruif, 2008; Huppertz et al., 2007; Mounsey et al., 2005), and increased stability of transglutaminase-treated casein micelles against disruption upon removal of hydrophobic interactions or removal of micellar calcium phosphate (Huppertz & de Kruif, 2008; Huppertz et al., 2007; Moon et al., 2009; Smiddy et al., 2006).

Table 5. Enzymatic cross-linking of caseins.

Enzyme*	Substrate	Mediator	Cross-linking	References
TG	Skim milk	-	Efficiency of cross-linking: κ-casein>β-casein>α-casein	Jaros et al. (2010); Sharma et al. (2001)
	Sodium caseinate	-	Efficiency of cross-linking: β-casein>α-casein>κ-casein	Jaros et al. (2010)
	Micellar casein or sodium caseinate dispersions in milk serum	-	Degree of polymerisation was higher in caseinate compared to micellar casein dispersion.	Bönisch et al. (2004, 2007a)
Tyrosinase	β-casein	-	Complete oligomerization with high enzyme dosage	Monogioudi et al. (2009)
	β-casein	-	Only *T.reesei* tyrosinase cross-linked caseins	Mattinen et al. (2008b)
	α_s-caseins	L-dopa (negative effect)	Almost complete oligomerization obtained with *T.reesei* tyrosinase	Selinheimo et al. (2007b)
Tyrosinase & laccase	α_s-caseins	FA & *p*-CA	Both enzymes induced cross-linking (mediators increased laccase efficiency)	Selinheimo et al. (2008)
TG & laccase	α_s-caseins	FA (for laccase)	Both enzymes induced cross-linking (laccase only with FA)	Steffensen et al. (2008)

*see the reference for the origins of the enzymes

Table 6. Enzymatic cross-linking of whey proteins.

Enzyme*	Substrate	Treatment	Cross-linking	References
TG	BLG	+/- high pressure	Cross-linking achieved only after high pressure treatment.	Lauber et al. (2001)
	WPI	pH (6–8) +/- heat treatment	Without heat treatment BLG was inert but slightly cross-linked at pH 8.0. ALA was cross-linked in all conditions.	Eissa et al. (2004); Eissa & Khan (2005)
	WPI	+/- DTT	BLG was cross-linked only in the presence of DTT whereas ALA was cross-linked in both conditions.	Faergemand et al. (1997); Færgemand & Qvist (1999)
Tyrosinase	BLG, ALA	pH (3–8) (CaffA used as mediator)	Both proteins were cross-linked to some extent with AbT (optimum at pH 4–5). ALA was cross-linked without the mediator.	Thalmann & Lötzbeyer (2002)
	BLG	alkaline (pH 9.0)	Some cross-linking with *T.reesei* tyrosinase only in alkaline conditions.	Partanen et al. (2011)
TG & laccase	BSA, BLG	native (FA used as mediator)	No cross-linking with TG. Laccase cross-linked both proteins when used with FA.	Steffensen et al. (2008)
Laccase & Tyrosinase	BSA, BLG	native	No cross-linking	Mattinen et al. (2006); Mattinen et al. (2008b)
Laccase	WPI	DTT (ChA was used as mediator)	ALA was more efficiently cross-linked than BLG. Extent of cross-linking was limited compared to TG.	Faergemand et al. (1998)

*see the reference for the origins of the enzymes

1.4 Structure modification of acid milk gels by enzymatic cross-linking

There are a vast number of studies showing the effects of TG-induced cross-linking of milk proteins on the structure of set and stirred types of acid milk gels prepared by yoghurt starter bacteria or chemical acidifiers (Jaros et al., 2006a). It has mainly been reported that introduction of a small number of covalent cross-links by TG-treatment can lead to milk protein gels with increased gel strength, increased elasticity and less syneresis (reviewed by Buchert et al., 2010; Huppertz, 2009; Jaros et al., 2006a). Acid-induced gels from TG-treated caseinate or milk have been reported to attain a homogeneous microstructure with finer network and smaller pores as compared to untreated gels (Færgemand & Qvist, 1997; Lorenzen et al., 2002; Myllärinen et al., 2007; Partanen et al., 2008;

Schorsch et al., 2000). The small pore size and homogeneity of the network was correlated to the superior water holding properties of yoghurt from TG-treated milk (Lorenzen et al., 2002). Gel preparation, enzyme treatment conditions and final effects of TG treatment on the structural properties of set-type milk and caseinate gels are summarised in Table 7 and 8, respectively.

However, extensive cross-linking, was found to result in impaired gel firmness due to restriction of proper rearrangements during gel formation (Jaros et al., 2010; Jaros et al., 2006b). In traditional yoghurt production, the effects of TG or TG-modified proteins on the activity of starter bacteria have also been investigated. TG-treatment of milk prior to starter culture addition results in prolonged fermentation times, as the small peptides needed by starter bacteria are not available due to cross-linking (Lorenzen et al., 2002; Ozer et al., 2007). This causes a negative effect on the aroma profile and acidity of yoghurt prepared from TG-treated milk, which can however be improved by concomitant addition of TG and starter bacteria. With optimized TG dosages, non-fat yoghurt with improved physical and sensory properties that are comparable to those of full-fat yoghurt could be produced without the need for additional protein or stabilizer (Ozer et al., 2007). TG-treatment of skim milk prior to fermentation was also shown to improve rheological properties and water retention in stirred type yoghurts even at reduced protein contents (Bönisch et al., 2007b, 2007c).

Currently there are only a few published reports on the possible effects of cross-linkages created by oxidative enzymes on the structural modification of acid milk gels. Yamaguchi (2002) reported increased viscosity and improved gelling ability of milk proteins after laccase treatment. Recently, Hiller & Lorenzen (2009) reported increased viscosity of milk due to laccase (together with ChA) and glucose oxidase treatments. Mushroom tyrosinase was reported to increase the viscosity of heat-induced milk protein gels prepared by addition of alginic acid and high-shear homogenization (Onwulata & Tomasula, 2010).

Table 7. Effects of TG treatment on the structure of acid-induced milk gels.

Gel preparation	Effects on final gel	References
• Heat-treated milk (92°C, 5 min) was incubated with TG. • TG was either inactivated or not prior to acidification. • Starter bacteria, 43°C.	• Increased gel strength and reduced whey separation (enhanced effect in skim milk and when TG was not inactivated). • More homogeneous microstructure with smaller pores. • Increased creamy perception in skim milk yoghurt.	Lorenzen et al. (2002)
• Pasteurized milk treated with TG under high pressure. • TG was inactivated prior to acidification. • GDL, 30°C.	• Substantial increase in G' when pressure and TG were applied simultaneously. • Reason: availability of whey proteins for cross-linking during high pressure treatment.	Anema et al. (2005)
• Raw skim milk was incubated with TG. • TG was inactivated prior to acidification. • Starter bacteria, 42°C.	• Increased breaking strength in gels from TG-treated milk. • 10–30% of casein oligomerization was sufficient to cause considerable effect on gel firmness.	Lauber et al. (2000)
• Heat-treated milk (90°C, 5 min) was incubated with TG. • TG was either inactivated or not prior to acidification. • Starter bacteria, 43°C.	• Increased viscosity and reduced whey separation during cold storage (enhanced effect when TG was not inactivated). • Growth of starter bacteria was negatively affected, causing slow acidification and acetaldehyde production.	Ozer et al. (2007)
• UHT milk was incubated with TG. • TG was either inactivated or not prior to acidification. • GDL, 30°C.	• TG did not affect G', whereas an increased elasticity and rupture force was attained in penetration tests. • At high TG dosages, formation of some physical interactions might be limited.	Jaros et al. (2006b)
• Native calcium phosphocaseinate was dissolved in milk salt buffer. • TG was not inactivated after incubation. • GDL, at 20 °C or 50 °C (slow acidification kinetics).	• Decreased gel formation time and increased G' was attained with TG-treatment. • TG treatment reduced the spontaneous syneresis. • Small mesh-sized microstructure with smaller aggregates was attained with TG-treatment.	Schorsch et al. (2000)

Table 8. Effects of TG-treatment on acid-induced caseinate gels.

Gel preparation	Effects on final gel	References
• Sodium caseinate solution (2.7 and 4.5%). • TG was added simultaneously with GDL. • Acidification at 4, 22, 37 and 50°C.	• Increased gel firmness with TG at all acidification temperatures. • Finer and more homogeneous microstructure in TG-treated gels.	Myllärinen et al. (2007)
• Acid casein solution (5%) was incubated with TG. • Acidification at 40°C, at varying GDL dosages.	• Gel firmness was found to be linearly related to the TG dosage but passed through a maximum with increasing GDL dosage.	Menéndez et al. (2004)
• Sodium caseinate solution (2.7 and 7.2%). • TG was added simultaneously with GDL. • Both acid and neutral gels were prepared at 22 or 50°C.	• Increased gel firmness with TG in acid gels. • At high caseinate concentration, TG-induced formation of a gel in neutral conditions was observed. • Diminished spontaneous syneresis in acid gel with TG (50°C). • No water separated from neutral (TG) gel upon cutting.	Partanen et al. (2008)

Tailoring of food structures, in this case fermented dairy products, by use of enzymatic protein cross-linking has been investigated for a few decades now. Research indicates that correct assessment of the structure modification by introducing intra- and/or inter-molecular covalent bonds to milk proteins necessitates thorough understanding of the molecular and the colloidal aspects of milk proteins and interactions during gelation. Extensive research has been conducted by using TG as a protein cross-linker, and molecular and macromolecular effects have been documented. The use of other enzymes, such as the oxidative enzymes laccase and tyrosinase for tailoring dairy gel structures should be explored in more detail, as they have shown promising results in cross-linking milk proteins.

2. Aims of the study

Research on the mechanism of enzymatic structure engineering of dairy products has mainly been carried out with transglutaminase. Currently there are only a few published reports on possible effects of oxidative enzymes on the structure of milk products. Investigation of the enzymatic cross-linking phenomena using different enzymes with dissimilar reaction mechanisms and target amino acid residues would enable better characterization of the role of such covalent linkages in protein-stabilized systems. Evidently, understanding of the molecular structure of the substrate proteins and their colloidal associations is necessary in order to interpret the impact of enzymatic cross-linking on the protein functional properties.

The present work aimed to identify the effects of protein cross-linking attained by different enzymatic pathways on the structure of acid-induced milk protein gels. More specifically, the aims were:

- To investigate laccase-, tyrosinase- and transglutaminase-induced cross-linking of caseins and whey proteins directly in milk or in solution, and to compare the actions of tyrosinase and transglutaminase on beta-lactoglobulin at different molecular and colloidal states in order to understand the structural constraints limiting inter-molecular cross-linking (Publication I).
- To compare the abilities of laccase, tyrosinase and transglutaminase to modify the rheological properties and the microstructure of acid-induced milk gels in which mainly intra-micellar cross-linking takes place or caseinate gels in which the colloidal state of the proteins is different from that in milk (Publication II and III).
- Finally, to investigate the mechanism behind improved mechanical and water holding properties of transglutaminase-treated acid milk gels (Publication IV).

3. Materials and methods

A brief description of the materials and methods used in the study is presented below. More detailed information can be found in the relevant publications (I–IV).

3.1 Materials

Enzymes, raw materials and some of the chemicals used in the study, as well as information on their origin or where they were used (when applicable) are listed in Table 9.

3.2 Characterization of the substrate protein conformation (Publication I)

Changes in the secondary and tertiary structure of BLG subjected to various pH conditions (pH 6.8–9.0) and heat treatments (80°C, 30 min or 125°C, 2 min) were assessed by circular dichroism (CD) spectroscopy. Far-UV (180–260 nm) and near-UV (240–330 nm) CD spectra were recorded using a CD spectropolarimeter (JASCO J710, Jasco Ltd., Japan).

3.3 Analysis of protein cross-linking (Publications I, II, III, IV)

Changes in the molecular weight and electrophoretic mobility of milk proteins after enzyme treatments were mainly followed by SDS-PAGE under reducing conditions (Publications I, II, III). In cases in which an acid-induced gel was prepared from enzymatically modified proteins (Publications II, III), the samples for SDS-PAGE were prepared from freeze-dried and re-dissolved gel samples. For β-lactoglobulin solutions, the state of aggregation via disulphide bridges after different pH and heat-treatment conditions was analysed by non-reducing SDS-PAGE (Publication I).

Table 9. Description of the materials used in the study.

	Information	Publication
Enzymes		
Laccase	Origin: *T.hirsuta*. Produced and purified at VTT[a]	II
Tyrosinase	Origin: *T. reesei*. Produced and purified at VTT[b] Origin: *A. bisporus*. Commercial preparation (Fluka Biochemica, Taufkirchen, Germany)	I, III
Transglutaminase	Origin: *S. mobaraensis*. Activa® WP or MP (Ajinomoto, Inc., Japan). Used as such or as further purified from non-protein ingredients [c]	I, III, IV
Raw materials		
Sodium caseinate	Commercial powder supplied by Valio Ltd. (Helsinki, Finland). Solution (5% or 3% (w/w)) was prepared in distilled water by mixing overnight at 4°C	II, III
Skim milk	Raw skim milk was supplied by Valio Ltd. Used as such, or after heat treatment at 90°C, 5 min.	III
Skim milk powder	Low-heat skim milk powder was supplied by Valio Ltd. Reconstituted (3.3% protein) in ultrapure water by constant stirring at 50°C, 1 h. Used after heat treatment at 80°C, 30 min.	IV
β-Lactoglobulin	Commercial powder (Sigma, St. Louis, MO, USA). Purity: 90% by PAGE, mixture of A and B variants. Used without further purification.	I
Chemicals		
GDL	Used for acidification of milk or caseinate solution. Hydrolyses gradually into gluconic acid.	II, III, IV
Ferulic acid	Used as a mediator in laccase reactions. Also used as the substrate in laccase activity assay at pH 7.0.	II
ABTS	Used as the substrate in tyrosinase activity assay at pH 4.5[d].	
L-Dopa	Used as the substrate in tyrosinase activity assay[e].	I, III
N-carbobenzoxy-L-glutaminylglysine	Used as the substrate in transglutaminase activity assay[f].	I, III, IV
Rhodamin B	Used for protein staining in microscopical analysis.	II, III, IV

According to; [a]Rittstieg et al., 2002, [b]Selinheimo et al., 2006, [c]Lantto et al., 2005, [d]Niku-Paavola et al., 1988, [e]Robb, 1984, [f]Folk, 1970

Size exclusion chromatography (SEC) using an ÄKTA purifier liquid chromatography system together with a Superdex 200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to elucidate the formation of covalently linked casein oligomers upon enzymatic cross-linking (Publication II).

The outcome of enzymatic reactions was also indirectly monitored via rheological measurements (Publications I, II, III, IV).

3.4 Preparation of acid-induced sodium caseinate or milk gels (Publications II, III, IV)

Sodium caseinate or milk samples were incubated with the selected enzymes prior to acidification. Acidification was performed by addition of GDL to enzyme-treated (or untreated in the case of controls) samples after temperating to the acidification temperature. Only set-type gels were studied. An overview of the enzyme dosages and other conditions is presented in Table 10.

Table 10. Enzyme pre-treatment and acidification conditions used in the preparation of acid-induced gels.

Pub.	Substrate	Enzyme	Enzyme dosage (nkat g^{-1} protein)	Enzyme treatment conditions	Acidification temperature (°C)
II	Sodium caseinate	Lac[a]	2.5, 25[b]	2h at RT or 45°C	25°C
III	Sodium caseinate	TrT, AbT TG	100 25	1 h at 40°C	30°C
	Milk				
IV	Reconstituted milk	TG	100	1 h at 40°C	20°C, 30°C, 40°C

[a] with/without 2.5 mM FA, [b]Based on activity on FA at pH 7.0

3.5 Structural properties of the acid-induced gels (Publications II, III, IV)

The gel formation dynamics and the properties of the final gel structures were analysed by rheological, microscopical, light scattering and gravimetric (syneresis) techniques. The techniques and the reasons why they were used are listed in Table 11. For all methods, a full description and the parameters used in this study can be found in the relevant publication. Brief descriptions of the rheological methods as they were generally utilized in the publications are given below.

Table 11. Methods used to characterize the gel structures.

Technique	Aim	Publication
Gel formation		
Small deformation oscillatory measurements (Rheometer)	Determination of viscoelastic properties (G', G'') during gel formation. The point of gel formation was also detected.	II, III, IV
Near-infrared light backscattering (Turbiscan®)	Monitoring of the change in aggregate size during gel formation. The point of gel formation could also be detected.	IV
Gel firmness		
Small deformation oscillatory measurements	Final values of the G' and tanδ indicate the firmness and the elasticity of the gel network.	II, III, IV
Large deformation (penetrating probe measurements)	The force at which the gel fractures and the distance that the probe travels until the fracture point indicates the firmness and elasticity (or brittleness) of the gel, respectively.	II, III, IV
Gel microstructure		
Confocal laser scanning microscopy (CLSM)	Analysis of the gel microstructure during and at the end of the gel formation. Rhodamine B was used to stain the proteins.	II, III, IV
Scanning electron microscopy (SEM)	Analysis of the gel microstructure with a higher resolution compared to CLSM. Size of the gel particles and the extent of particle-particle interactions in the gel network are better observed.	III
Water retention properties		
Spontaneous syneresis (gravimetric)	Measurement of the water (or whey) released from the gel without any external force applied.	IV
Water holding capacity (gravimetric)	Measurement of the water (or whey) retained in the gel after moderate centrifugation.	IV

3.5.1 Small deformation oscillatory measurements

Small deformation oscillatory measurements allow determination of the viscoelastic properties of acid-induced milk gels during gel formation and ageing without disturbing the sample structure. Implications about the number and strength of bonds or structural changes can be inferred from the measured parameters.

During a time sweep, an oscillating (sinusoidal) stress (σ) or strain (γ) is applied to the sample at constant frequency (ω) and oscillating response; either the strain or stress, respectively is measured. When a sinusoidal strain is applied, the amplitude of the stress response and the delay in the phase angle (δ) will depend on the viscoelastic properties of the sample. In a viscous material, most of the

stress is dissipated as heat due to internal friction created in the material, whereas in the case of an elastic material the stress is transmitted through the material. Accordingly, for a solid, $\delta = 0°$ (in-phase), for a pure liquid $\delta = 90°$ (out-of-phase) and for a viscoelastic material, δ would be between 0° and 90°. In the linear viscoelastic region (LVR), the sinusoidal shear stress response is given by:

$$\sigma = \sigma_0 \sin(\omega t + \delta) \tag{3.1}$$

where σ_0 is the shear stress amplitude. Equation 3.1 can be rewritten as:

$$\sigma = \underbrace{\sigma_0 \cos\delta \sin\omega t}_{\text{in-phase response}} + \underbrace{\sigma_0 \sin\delta \cos\omega t}_{\text{out-of-phase response}} \tag{3.2}$$

Accordingly, frequency dependent shear elastic (storage) modulus (G') and the viscous (loss) modulus (G'') are defined as follows:

$$G'(\omega) = \frac{\sigma_0 \cos\delta}{\gamma_0} \tag{3.3}$$

$$G''(\omega) = \frac{\sigma_0 \sin\delta}{\gamma_0} \tag{3.4}$$

Finally, stress response of a sinusoidal strain applied to a viscoelastic material (in the LVR) can be written as follows:

$$\sigma = G'(\omega)\gamma_0 \sin\omega t + G''(\omega)\gamma_0 \cos\omega t \tag{3.5}$$

G' is a measure of the energy stored or recovered during one oscillation cycle, and thus it reflects the elastic character whereas G'' is a measure of the energy lost or dissipated and thus reflects the viscous character of the gel. A higher value of G' compared to G'' indicates that the material shows predominantly elastic character (Compiled from; Gunasekaran & Ak, 2000; Miri, 2011; Rao, 1999). Another parameter commonly used is tanδ which corresponds to:

$$\tan\delta = \frac{G''}{G'} \tag{3.6}$$

In a gelling system, the point of gel formation can be defined as the point at which tanδ = 1 (G' = G'') (Horne, 1999). After that point tanδ would decrease steeply, indicating the domination of elastic character and the formation of a gel network. The gelation point can also be defined in other ways. For example, it is often considered to be the point at which G' > 1 or G' > 0.1 (depending on the gel characteristics).

In this study, formation of acid-induced sodium caseinate and milk gels was followed by small deformation oscillatory measurements performed with a stress-controlled rheometer (StressTech, Rheologica Instruments AB, Lund, Sweden). The experimental parameters were as follows; frequency: 1.0 Hz, strain: 0.01 for 5% sodium caseinate solution (Publication II) and frequency: 0.1 Hz, strain: 0.01 for 3% sodium caseinate solution or milk (~3.3% protein) samples (Publications III and IV).

When the desired final pH values were achieved, strain sweeps (to determine the linear viscoelastic region or the maximum strain the sample can withstand without loss of structure) and frequency sweeps (to further elucidate the elastic character of the gel) were performed.

3.5.2 Large deformation tests

The firmness and elasticity of the gels were also measured by a penetrating probe test performed with a texture analyser (TA-HDi, Stable microsystems, Ltd., Godalming, England). In this method, a plastic probe is immersed with a constant low speed into the gel sample to a pre-defined depth. The force at which the gel fractures (a sudden decrease in force) gives an idea about the firmness of the gel. The distance the probe travels before the fracture point is reached indicates the elasticity or brittleness of the gel sample. The area under the force vs distance curve is also taken as a measure of gel firmness. Similar to small deformation oscillatory measurements, the large deformation properties of a gel are also affected by the number and type of the bonds and their spatial distribution (Lakemond & van Vliet, 2008b). For example, the force needed to break a strand of gel particles held together by covalent bonds is higher compared to one with non-covalent bonds but the distance (strain) to the fracture point is not directly related to the bond strength but more to the curvature of the strand as it needs to be straightened before it can be ruptured (Lakemond & van Vliet, 2008b).

In this study, a hemispherical plastic probe (∅ 1.27 cm) was used with a constant speed of 0.5 mm s^{-1}. The probe was immersed to 70% of the gel height. All measurements were performed at room temperature (Publications II, III, IV).

3.6 Interfacial shear rheology (Publication I)

Interfacial shear rheology of adsorbed β-lactoglobulin layers was performed in order to gain insight in to the formation of intra- and/or intermolecular covalent linkages upon addition of tyrosinase or transglutaminase beneath the interfacial film. Shear deformations applied at the interfacial layers provide indirect information on the interactions between the adsorbed protein molecules (Krägel & Derkatch, 2010). Interfacial layers are in general weak, and thus the rheometers used should have a high torque and displacement sensitivity. Furthermore, the geometry used must not have a high inertia that would dominate the measurement. The Du-Noüy ring is an advantageous geometry in terms of its light weight, although, its poor

interfacial pinning and the difficult-to-calculate flow field along the ring are the weak points of the system as such (Krägel & Derkatch, 2010).

Small deformation oscillatory measurements at the air-water interface were performed with a stress-controlled rheometer (AR-G2, TA Instruments, Crawley, West Sussex, UK) equipped with a Pt-Ir du-Noüy ring (13 mm diameter). The sample (BLG solution) was placed in the sample dish and the du-Noüy ring was quickly placed at the interface between the air and the protein solution (as shown in Figure 4A). When it is oscillated sinusoidally with a pre-defined strain amplitude, the adsorbed protein film is sheared analogously to bulk systems. In this study, surface shear moduli were followed at a frequency of 0.1 Hz and a constant strain of 0.5%, which was measured to be in the LVR. The protein adsorption and film formation were followed for 1 h for BLG alone before injection of the enzymes under the surface (Figure 4A). In this way, a representative film was formed and the subsequent effect of any enzymatic action could be assessed (Publication I).

The surface pressure of adsorbed BLG layers was also measured in order to obtain insight into the adsorption rate of the BLG and saturation level of the interface. Moreover, possible desorption during enzyme injections or disturbances after enzyme addition were also followed. Surface pressure was measured with a KSV film balance (Minimicro series, KSV Instruments) using a platinum Wilhelmy plate at room temperature. All the conditions were maintained similar to those of the interfacial rheology (as shown in Figure 4B) with the aim of creating the same adsorbed protein layer (Publication I).

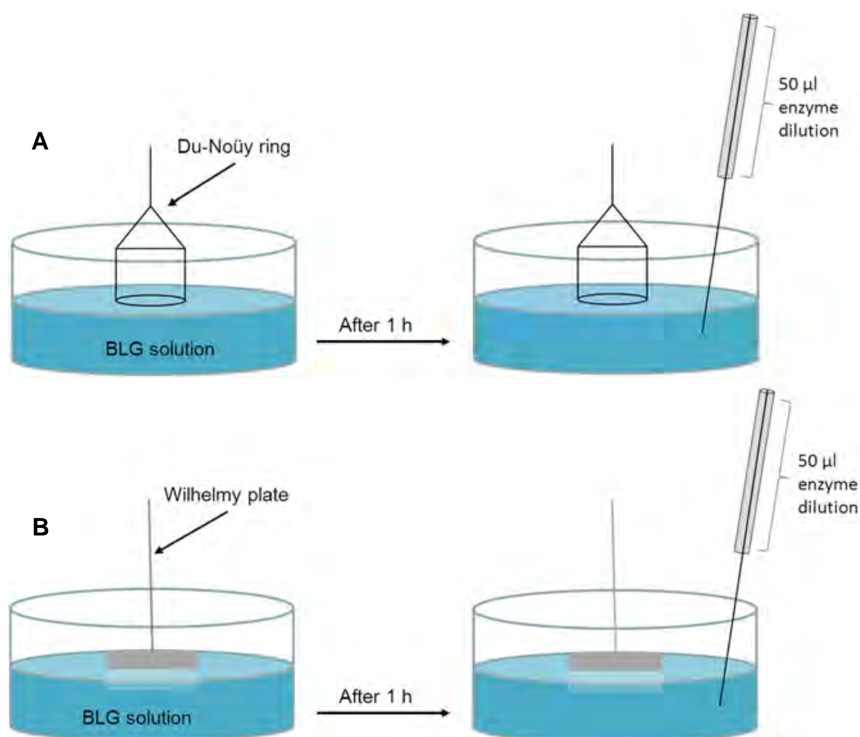

Figure 4. Representation of the interfacial shear rheology (A) and surface pressure (B) measurements which were performed simultaneously (Publication I).

3.7 Statistical analysis

Results presented in this study are expressed as mean values. One-way ANOVA followed by an appropriate comparison of means test was used when necessary (SPSS 14.0, SPSS Inc., Chicago, IL, USA). Values were considered to be significantly different at $p<0.05$.

4. Results and discussion

4.1 Cross-linking of milk proteins

4.1.1 Cross-linking of caseins

The cross-linking ability of *T. hirsuta* laccase (Lac), *A. bisporus hirsuta* tyrosinase (AbT), *T. reesei* tyrosinase (TrT) or transglutaminase (TG) with milk proteins was studied in milk or sodium caseinate solution. In milk, protein cross-linking was not observed as a result of AbT treatment either in raw or heat-treated (90°C, 5 min) milk, whereas TrT cross-linked milk proteins in both cases (Figure 5A). Mainly β-casein was cross-linked, followed by α_s- and κ-caseins (the monomer bands were not diminished in any of the caseins). The two tyrosinases have been reported to have differences in their cross-linking ability (Mattinen et al., 2008b; Selinheimo et al., 2007b). Accordingly, TrT is capable of cross-linking caseins as such, whereas AbT needs an auxiliary low molecular weight phenolic compound for cross-linking (Selinheimo et al., 2007b). TG did not cross-link proteins in raw milk, although extensive protein cross-linking was observed in heated milk (Figure 5A). This was an expected outcome due to the presence of a heat labile low molecular weight inhibitor of TG in bovine milk serum (Bönisch et al., 2007a; de Jong et al., 2003). Milk was also treated with laccase (Figure 5B). Laccase treatment caused formation of faint oligomer bands, with a decrease in intensity of casein monomers in both raw and heated milk. However, a clear fragmentation band below 18 kDa was observed in both samples (Figure 5B). The laccase preparation was shown to have a low protease contamination (Publication I). However, the protein fragmentation could also be directly caused by the laccase reaction mechanism. Highly reactive free radicals formed as a result of the oxidation by laccase can easily undergo non-enzymatic reactions leading to protein fragmentation as well as protein cross-linking (Claus, 2003; Thurston, 1994). Protein fragmentation due to laccase treatment has been reported for chicken breast myofibril proteins by Lantto, et al. (2005) and for wheat flour doughs by Selinheimo, et al. (2007a).

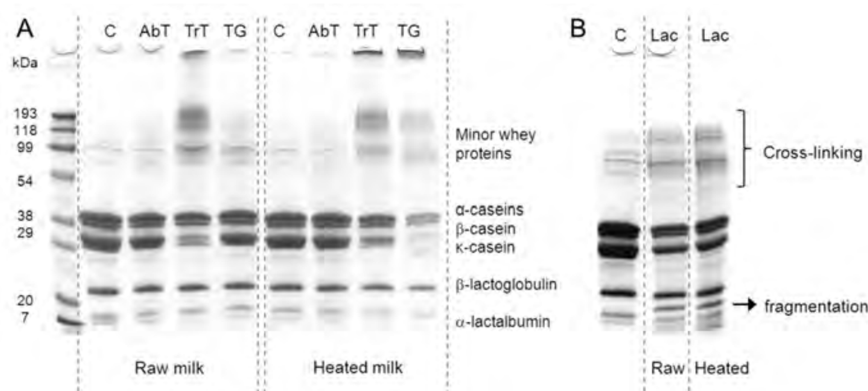

Figure 5. SDS-PAGE analysis of enzyme-treated raw or heated (90°C, 5 min) milk. The milk was incubated with AbT, TrT, TG (A) and Lac (B) for 1 h at 40 °C. The enzyme dosage was 500 nkat g^{-1} of protein for all enzymes (unpublished data).

The extent of protein cross-linking by laccase can be increased by the use of a low molecular weight phenolic compound as mediator. A dilute solution of sodium caseinate was treated with laccase with or without FA (Figure 6). Incubation of sodium caseinate with laccase caused the formation of high molecular weight reaction products in increasing amounts as a function of increased enzyme dosage. The intensity of the oligomer bands was increased when laccase was used together with FA. Similarly, the intensity of the degradation band was also increased with increasing enzyme dosage or by addition of FA. The effect of enzyme dosage on degradation band intensity indicates protease contamination in the laccase preparation used, whereas the increased intensity of the degradation band with FA treatment suggests that the degradation band may also be caused directly by radical-induced reactions.

Due to their rheomorphic conformation, caseins are not heat sensitive proteins. Their flexible structure results in the formation of inter-molecular cross-links without any need for pre-treatment. However, the compact globular structure of whey proteins limits the possibilities for enzymatic catalysis in their native state, necessitating a pre-treatment for a change in fold. There was no clear change in the intensity of the β-lactoglobulin or α-lactalbumin bands in raw milk (Figure 5). In heated milk, TG-treated sample showed a decreased intensity of whey protein bands (Figure 5A). The effect of heat treatment on cross-linking of β-lactoglobulin is elucidated in Section 4.1.2.

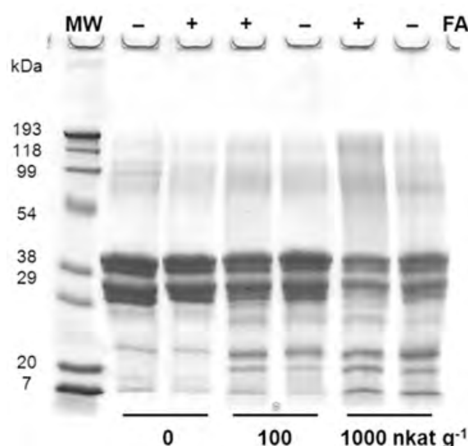

Figure 6. Cross-linking of 0.3% sodium caseinate solution (in 0.1M NaP buffer at pH 7.0) with *T. hirsuta* laccase (Lac) with or without 2.5 mM FA at room temperature for 2 h. (Enzyme activity based on oxygen consumption on 10 mM FA at pH 7.0) (unpublished data).

4.1.2 Cross-linking of β-lactoglobulin (Publication I)

Enzymatic modification of BLG was first investigated by using TG, TrT, AbT and Lac, all at an enzyme dosage of 1000 nkat g^{-1} protein (Figure 7). Without any treatment (at pH 8.0), only TG could cause cross-linking of BLG monomers into dimers and trimers (Figure 7A). After heat treatment (80°C, 15 min), both TG and TrT were able to cross-link the BLG molecules (Figure 7B). The effect was more pronounced in the case of TG. The other two enzymes, AbT and Lac, were unable to catalyse protein cross-linking as such. However, concomitant use of caffeic acid (CafA) resulted in considerable oligomerization of BLG (data not shown).

Based on the observations in Figure 7, an attempt to link the secondary and tertiary structural properties of BLG to its proneness to enzymatic cross-linking by TrT and TG was made (Publication I). The molecular conformation of BLG at different pH values (pH 6.8, 7.5, and 9.0) before and after the heat-treatment (80°C, 30 min) was determined by CD spectroscopy. Enzymatic modification of the BLG molecules in the same conditions was analysed by SDS-PAGE. The lowest pH, 6.8, was chosen as a reference as it is the physiological pH in which BLG is found naturally in dimeric form, and closer to its native-folded state. The pH values 7.5 and 9.0 were selected as they represent the Tanford transition (Tanford et al., 1959) and the start of irreversible alkaline denaturation (Taulier & Chalikian, 2001), respectively. From neutral to alkaline pH, the monomer-dimer equilibrium shifts to monomeric form, exposing the dimer interface. Despite the increased negative charge, the partially unfolded monomers tend to associate (slow and time-dependant), driven by exposed hydrophobicity (Barteri et al., 2000; Partanen

et al., 2011). It was reported that a monomer-aggregate equilibrium might be attained at pH 9.0, depending on the protein concentration, ionic strength and temperature (Barteri et al., 2000). Besides the conformational changes which lead to increased reactivity towards enzymes, the pH-induced exposure of the dimer interface, the shifts in dimer to monomer and multimer equilibria and formation of physical and disulphide-linked aggregates upon heat-treatment are relevant factors in the formation of intra and/or inter-molecular cross-linking.

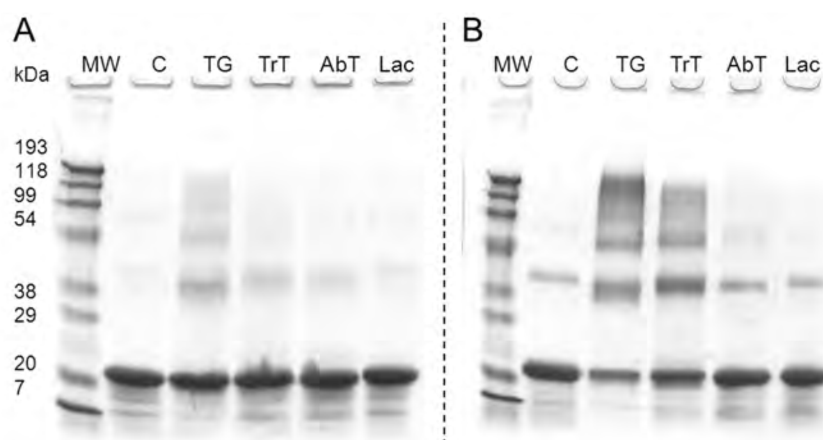

Figure 7. Cross-linking of β-lactoglobulin (1.7 mg mL^{-1} in 50 mM sodium phosphate buffer at pH: 8.0) at 40 °C for 20 h without any heat treatment (A) and after heat-treatment at 80 °C, 15 min (B). The enzyme dosage was 1000 nkat g^{-1} of protein for all enzymes. (Laccase reaction was carried out at pH 4.5, 50 mM acetate buffer) (unpublished data).

Without the heat-treatment, far-UV results showed β-sheet-dominant structures at all pH values studied, with a slight shift only at pH 9.0 (Figure 8A). In near-UV spectra, the intensity of the negative peaks at 293 and 285 nm, which are typical features in native conformation, was decreased at pH 9.0 as compared to neutral pH values. This indicated partial loss of the specific and rigid packing of aromatic residues, namely tryptophan and tyrosine, at pH 9.0 without any heat-treatment (Figure 8B). Upon heat treatment, a change in ordered secondary structure was detected at all pH values (Figure 8A). The shift in negative peak towards 200 nm indicated increasing random coil conformation with a loss in α-helical and β-sheet structures. The near-UV spectra of heat-treated BLG showed a significant loss of tertiary structure detected by diminishing of negative peaks and lower negative ellipticity values obtained at all wavelengths, which was more pronounced at pH 9.0 (Figure 8B).

Enzyme (TrT and TG)-induced cross-linking of BLG in the above conditions was analysed by reducing SDS-PAGE (Figure 9). Without heat-treatment, TrT did not induce any inter-molecular cross-linking at the studied pH conditions, whereas

TG-treatment could induce inter-molecular cross-linking at pH 7.5 (Figure 9A) and at pH 8.0 (Figure 7A). The only conformational change in BLG at around pH 7.5 that could explain this behaviour is a local displacement of the EF loop opening the calyx interior (Qin et al., 1998) and the increased hydration of the molecule, which does not cause global change in protein conformation (Taulier & Chalikian, 2001).

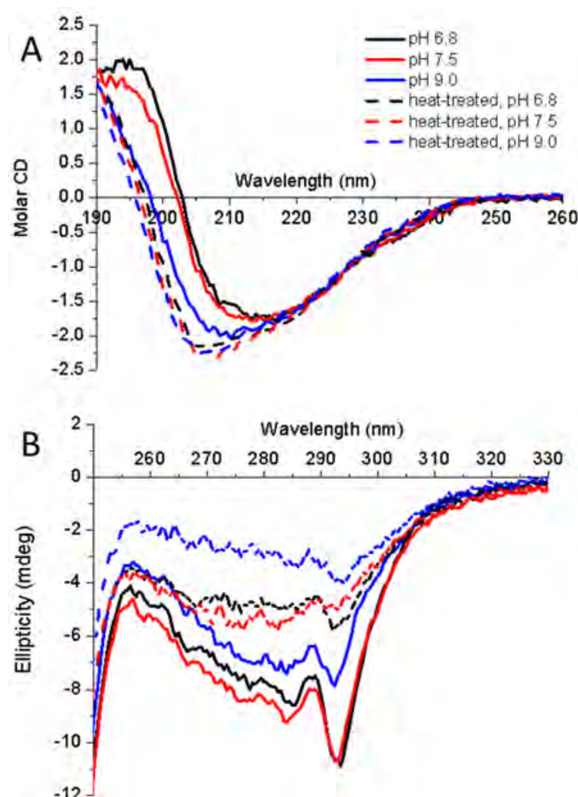

Figure 8. Far-UV (A) and Near-UV (B) CD spectra of non-heated (solid lines) and heated (80°C, 30 min) (dashed lines) BLG molecules at pH 6.8 (black), pH 7.5 (red), and pH 9.0 (blue) (Publication I).

At pH 9.0, inter-molecular cross-linking with TG was limited to smeared dimers only. The reason could well be the limited activity of TG at pH 9.0 (Lu et al., 2003). TrT was unable to induce any cross-linking at pH 9.0, despite a loss of tertiary structure. This inability could be due to the presence of only four tyrosine residues in BLG. Two of the tyrosines have been shown to be close to the surface and thus exposed in native state, but still somewhat hindered. Two other tyrosines are buried in the hydrophobic core, only one being exposed upon partial denaturation (Brownlow et al., 1997; Townend et al., 1969). Thus, the number of accessible tyrosine residues might still be highly limited without further unfolding.

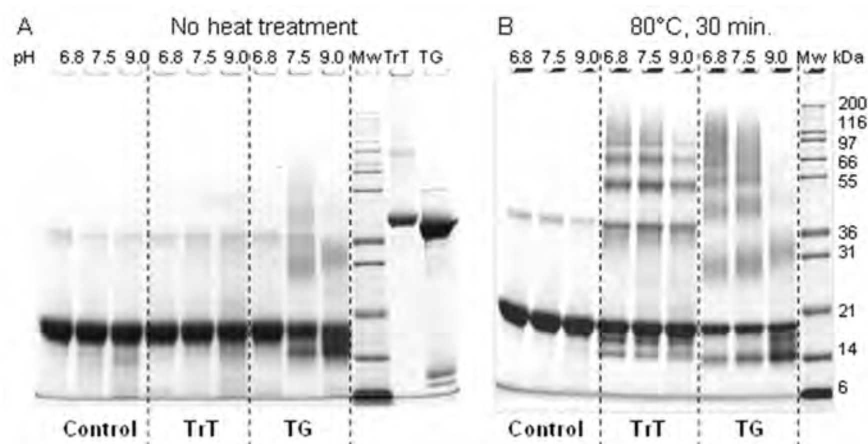

Figure 9. Reducing SDS-PAGE of BLG at pH 6.8, 7.5 and 9.0 without any heat-treatment (A) and after heat treatment at 80°C, 30 min. (B). BLG was cross-linked by 1000 nkat g^{-1} TrT and 1000 nkat g^{-1} TG for 18 h at RT and 40°C, respectively. The last two lanes (A) represent the enzymes loaded alone (Publication I).

Upon heat treatment, both enzymes induced formation of covalently linked BLG dimers, trimers and higher oligomers at all pH values (Figure 9B), which was complementary to the conformational changes detected after heat-treatment. An interesting outcome of the SDS-PAGE patterns was the difference observed between the shape and mobility of the cross-linked BLG bands with TrT and TG (Figure 9B). The broadening of the TG-induced oligomer bands could be attributed to heterogeneity of the created covalent bonds between and within the related BLG molecules due to the higher number of reactive residues for TG as compared to TrT. It should also be noted that in the absence of available lysines in close proximity of glutamines, TG action induces a deamidation reaction. As a consequence, glutamines are transformed into glutamic acid residues, which might even decrease the pI of BLG (Nieuwenhuizen et al., 2004), causing more heterogeneity. Increased band mobility could be due to extensive intra-molecular cross-linking of BLG molecules caused by TG, which probably affects the overall shape of the SDS-denatured molecule. Hellman et al. (2011) showed that an intra-molecularly cross-linked globular protein monomer showed delayed elution in size exclusion chromatography, indicating a more compact globular structure. Formation of tightly packed oligomers of β-casein with a smaller radius of gyration and more compact shape by TG but not by TrT was also previously shown (Monogioudi et al., 2009, 2011). Accordingly, the low molecular weight bands observed below the BLG monomers (~14 kDa) could also be due to intra-linked monomers with altered mobility.

Susceptibility of BLG to enzymatic cross-linking when adsorbed at the air-water interface was also analysed. Once adsorbed to interfaces, the BLG molecule has been reported to rearrange its conformation and even partially to unfold (Wilde, 2000), which may increase the accessibility of the target amino acid residues.

Interfacial shear rheology was utilized to obtain evidence for cross-linking of adsorbed BLG molecules. Surface pressure measurements revealed that at the studied BLG concentration (0.05 mg mL^{-1}), adsorption to the air-water interface was rather fast for both native and heat-treated (80°C, 30 min) BLG molecules. The surface pressure reached ~20 mN m^{-1} within seconds and did not change thereafter (see Figure 7 in Publication I). Even though the interface was saturated with BLG molecules instantly, G′ continued to increase with time (the first 1 h in Figure 10), implying on-going structural organization with increasing lateral interactions between adsorbed molecules. Transglutaminase was injected underneath the packed protein layer. After addition of TG to the sub-phase, the film first recovered from the disturbance created during the injection. After that, film strength was lowered, as was observed by decreasing G′ for both native and heat-treated samples (Figure 10). In the control samples, in which either buffer or inactivated enzyme preparation (Figure 10B) was injected instead of active TG, G' continued to increase after recovering from the injection damage and plateaued at a higher value. Overall, there was a negative effect of the enzyme treatments on development of G′, which was more evident when BLG was heat-treated.

The results indicate that inter-molecular cross-linking between the adsorbed molecules which would enhance the film strength was not achieved. Once adsorbed rapidly at such high surface concentration, BLG molecules attained a constrained structure. As a consequence, limited conformational re-organization prevented formation of enzyme-induced inter-molecular covalent links. In fact, Romoscanu & Mezzenga (2005) showed that glutaraldehyde-induced cross-linking increased the elastic modulus of non-densified BLG interface, whereas the effect was reversed when glutaraldehyde was applied on densified interface. On the other hand, intra-molecular links could still be formed within the adsorbed molecules. Intra-molecularly cross-linked globular proteins have been reported to be locked in their globular fold (Hellman et al., 2011). Formation of more compact structures was also shown by the altered mobility of the TG-treated oligomers on the SDS-PAGE gel (Figure 9B). Such bonds created by TG would then further impede the rearrangement of the adsorbed molecules during film ageing and lead to decreased physical protein-protein interactions. It has been reported that formation of compact molecules by TG action limits exposure of hydrophobic regions and thus attenuates hydrophobic interactions in whey proteins (Eissa & Khan, 2006).

The results emphasize the importance of structural and colloidal aspects of protein molecules in controlling inter- or intra-molecular bond formation by cross-linking enzymes.

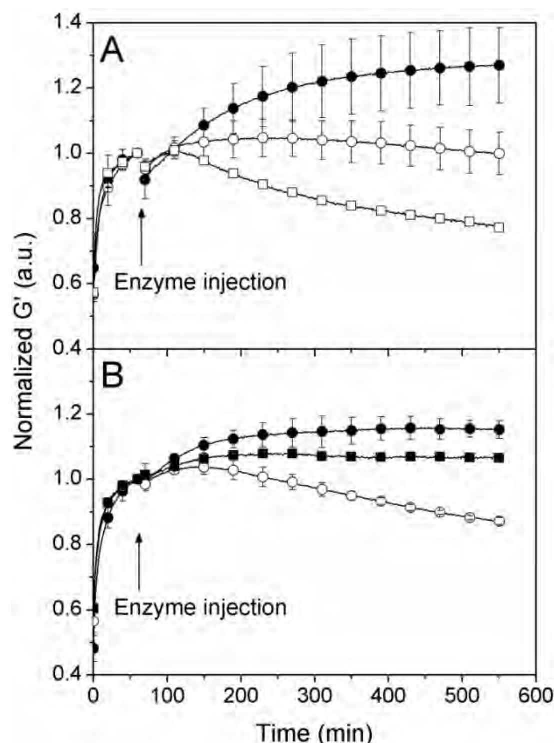

Figure 10. Effect of TG on interfacial shear elastic modulus (G') of non-heated (A); and heated (80°C, 30 min) (B) BLG solutions at pH 6.8. BLG adsorption was followed for 1h, after which 10 000 nkat g^{-1} TG was injected to the sub-phase (○). Only buffer was injected to the control samples (●). In addition, a curve with 50 000 nkat g^{-1} TG injection (□) is shown in (A), and a curve with inactivated TG injection (■) in (B). Vertical bars represent standard deviation. Values were normalized to the G' values at 60 min (immediately before enzyme or buffer injection) for each sample (Publication I).

4.2 Structural modification of acid-induced milk gels by protein cross-linking

4.2.1 Impact of laccase on the structure of acid-induced caseinate gels (Publication II)

Effects of laccase-induced modification of caseins on the rheological properties and the microstructure of acid-induced sodium caseinate gels were elucidated. *T. hirsuta* laccase was used with or without FA. The gel formation of laccase-treated sodium caseinate solutions was followed by small deformation oscillatory measurements during acidification. Storage modulus (G′) of the samples was

monitored at the acidification temperature of 25 °C for 5 hours (until ~pH 4.5). The final G′ refers to the G′ value attained after 5 h from GDL addition.

Laccase as such (without FA) did not result in increased final G′; infact, when used at the highest dosage, it caused a lower G' compared to the control (Figure 11A). However, when laccase was used together with FA, increase in final G′ as compared to the laccase-free control gel was observed for both laccase dosages (Figure 11B). The weakening of the gel strength observed when laccase was used as such (without FA) was attributed to the detectable protease activity in the laccase preparation, since no reduction in final G′ was observed when a protease-free laccase was used (see Figure 4 in Publication II). Other authors have also observed a negative effect of *T. hirsuta* laccase (at high dosage) on gelling of chicken-breast myofibril proteins (Lantto et al., 2005) and on the rheology of wheat bread dough (Selinheimo et al., 2007a). In both studies, it was reported that the negative effect of laccase on texture was not due to any protease activity but to the radical-induced mechanism of laccase. In fact, when the SDS-PAGE patterns of freeze-dried caseinate gels were analysed, a faint fragmentation band was still observed in the samples treated with the laccase preparation which was devoid of any detectable protease contamination (see Figure 1 and Publication II), supporting the role of radical-induced degradation.

Large deformation tests were performed with a texture analyser 22 h after GDL addition (at 25°C). The final pH of the gels was ~pH 4.2. The firmness of the gels treated with laccase in the absence of FA was similar to that of the laccase-free control regardless of the enzyme dosage (Figure 12A). Even though some cross-linking was observed at high laccase dosage without FA, the firmness of the gel was not increased. Trace protease activity detected in the laccase preparation probably altered the structure of the gel, counteracting the effects of the cross-links formed. When laccase was applied together with FA, the firmness of caseinate gels was significantly ($p<0.05$) increased compared to the control for both laccase dosages (Figure 12A).

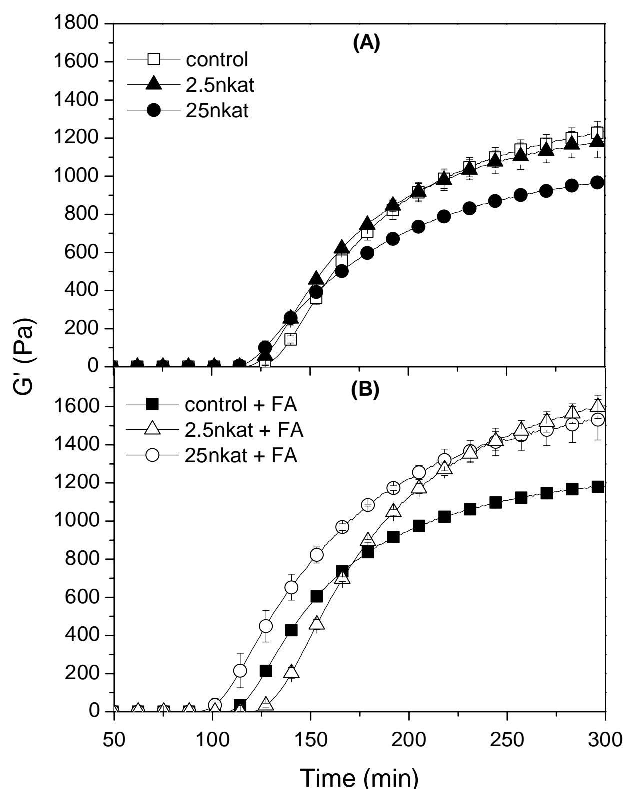

Figure 11. Effect of laccase on the storage modulus (G') of caseinate solution during acidification. Before acidification (with 1.13% GDL at 25 °C), sodium caseinate solutions were pre-treated for 2 h at 45 °C by: Laccase (A) or laccase + 2.5 mM FA (B) with the dosages; control without laccase and FA (□); control + FA (■); 2.5 nkat g^{-1} laccase (▲); 25 nkat g^{-1} laccase (●); 2.5 nkat g^{-1} laccase + FA (∆); 25 nkat g^{-1} laccase + FA (○). Vertical bars are the standard deviations at each data point (Publication II).

Microstructure of the final gels was also analysed and the micrographs are shown together with large deformation results in Figure 12. For all samples, the structure was homogenous, with casein aggregates linked together forming a small mesh-sized particulate gel network (Figure 12). When caseinate was pre-treated with a high dosage of laccase without FA prior to gel formation, a clear increase in pore size compared to the laccase-free control gel was observed (Figure 12D). Once again, the proteolytic activity could be the reason behind coarsening of the gel structure. When laccase was used together with FA, a finer and denser gel network was observed at both laccase dosages (Figure 12E and F), indicating the formation of stronger gels, which was in accordance with both small and large deformation measurements.

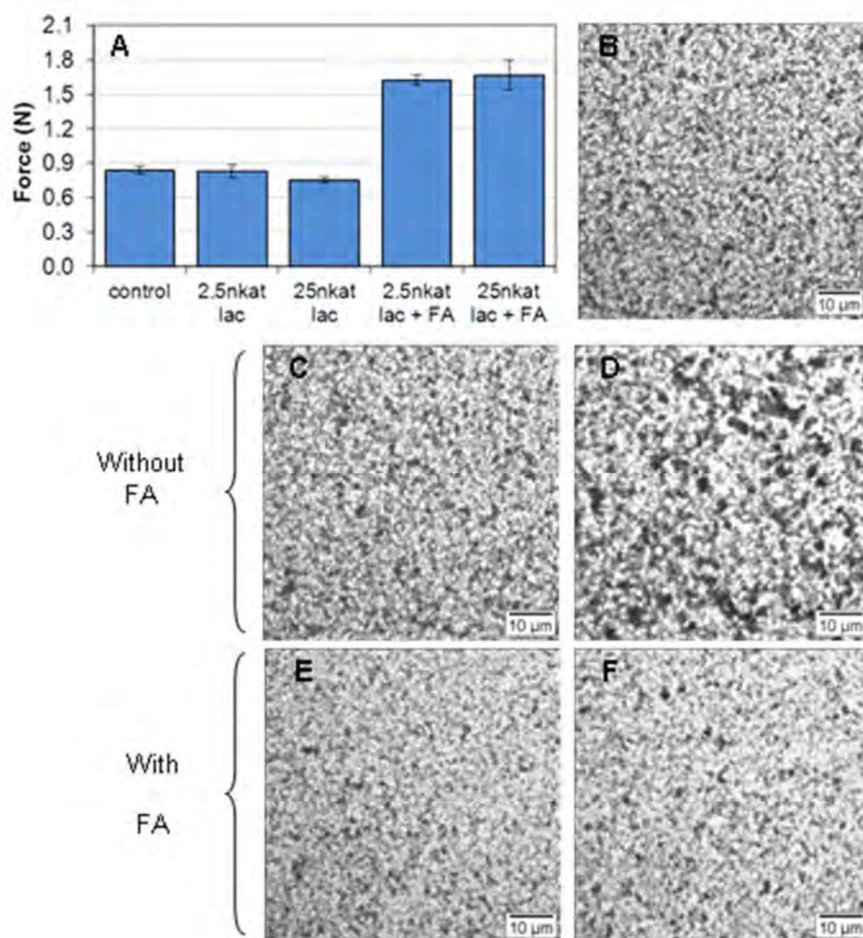

Figure 12. Firmness (force at rupture point) graph (A) and the CLSM images of sodium caseinate gels acidified at 25 °C with 1.13% GDL. Before acidification, sodium caseinate solutions were pre-treated by laccase for 2 h at 45 °C with the dosages; control without laccase and FA (B); 2.5 nkat g^{-1} laccase (C); 25 nkat g^{-1} laccase (D); 2.5 nkat g^{-1} laccase + FA (E); 25 nkat g^{-1} laccase + FA (F) (Publication II).

The results have shown that in laccase reactions the use of a mediator, in this case FA, significantly enhances inter-molecular protein cross-linking, leading to a finer microstructure and increased gel firmness in acid-induced caseinate gels. Even a small extent of casein oligomerization (see Figure 1 in Publication II) was sufficient to increase the gel strength significantly. Similarly, minor proteolytic activity in the laccase preparation also affected the gel properties. This should be taken into account when using commercial enzyme preparations. In addition, the radical-induced fragmentation may also have a role and needs further elucidation.

4.2.2 Impact of tyrosinases and transglutaminase on the structure of acid-induced milk gels (Publications III and IV)

The potential of AbT and TrT in structural engineering of acid-induced skim milk gels was studied and compared to that of TG. Raw or heat-treated (90 °C, 5 min) skim milk was pre-treated (1 h at 40 °C) with the enzymes prior to acidification.

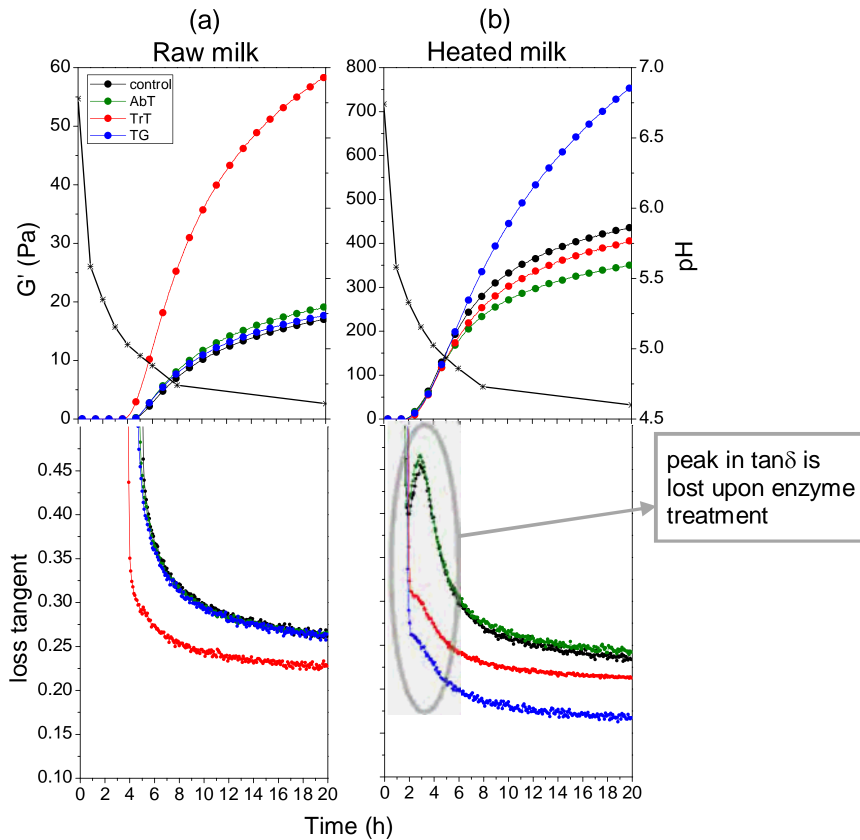

Figure 13. Storage modulus (G') and loss tangent of acidified raw milk (a) and heated (90 °C, 5 min) milk (b) gels. Before acidification, samples were treated by 100 nkat g^{-1} AbT, 100 nkat g^{-1} TrT, or 25 nkat g^{-1} TG, for 1 h at 40 °C. Acidification was performed at 30 °C, with 1.2% GDL. The change in pH during acidification of the control samples is also shown in the upper graphs (✳) (Publication III).

Oscillatory rheology was used to analyse the viscoelastic behaviour of the chemically acidified raw and heated skim milk gels. Storage modulus (G') of the samples was monitored during acidification at 30 °C for 20 h (to a final pH of 4.6) (Figure 13). The final G' refers to the G' value attained 20 h after GDL addition.

In raw milk (Figure 13a), final G' of the TrT-treated sample was three times higher than that of the enzyme-free control. Moreover, lower tanδ values were attained all through the acidification in TrT-treated milk compared to the control. On the other hand, AbT and TG treatments did not show any effect on G' or tanδ values. TrT was the only enzyme which resulted in protein cross-linking in raw milk (see Figure 2 in Publication III). The formation of inter-molecular covalent bonds by TrT before and during acidification resulted in increased gel firmness and elasticity.

Heat treatment at 90 °C led to increased gelation pH, (from pH 4.9 in raw milk to ~pH 5.4 in heated milk) and increased final G' of the milk gels compared to raw milk. TG treatment of heated milk led to an acid gel with considerably higher G' compared with the enzyme-free control, or with AbT- or TrT-treated gels. Neither of the tyrosinases showed a positive effect on G' in heated milk (Figure 13b). Furthermore, the value of tanδ was significantly lowered throughout the acidification in TG-treated sample. Interestingly, even though TrT treatment resulted in protein cross-linking comparable to TG (see Figure 2 in Publication III), there was no effect of TrT treatment on either the G' or the firmness detected by large deformation tests on the final gels (see Figure 4 in Publication III). However, similarly to TG, the TrT treatment of heat-treated milk resulted in decreased tanδ values during acidification. Furthermore, the local maximum (peak) in tanδ observed for control and AbT-treated gels around pH 5.1–5.2 was missing for TrT- and TG-treated samples (Figure 13b). The occurrence of the tanδ peak in the heated milk gels is attributed to loosening of the intra-micellar structure upon removal of CCP from the micelles (Anema, 2008, 2009a; Horne, 2003; Lakemond & van Vliet, 2008a; Lucey, 2002; Lucey et al., 1998b). The absence of the tanδ peak in the gels from TG- and TrT-treated milk is most probably due to intra-micellar covalent bonds created by both enzymes, leading to increased micellar integrity and hindering the weakening of intra-micellar structure upon solubilisation of CCP. Formation of highly stable (against removal of micellar calcium phosphate) casein micelles upon TG-induced intra-micellar cross-linking has been reported previously (Huppertz & de Kruif, 2008; Smiddy et al., 2006).

The microstructure of the gels was first visualised by CLSM. It was observed that gels from TrT- or TG-treated milks attained a finer microstructure with smaller pores compared to the control gel from heated milk (see Figure 5 in Publication III). With this technique, it was not possible to detect any differences between the microstructure of TrT- and TG-treated heated milk gels even though the rheological measurements showed a significant difference. Thus, the gel structures were compared at higher resolution attained by SEM. It was observed that TrT or TG treatment resulted in gel particles with smaller size compared to the non-enzyme treated control (Figure 14). SEM images also revealed that TrT did not improve the connectivity of the protein particles forming the gel. Protein particles were close to each other, like aggregates of individual particles. However, in TG-treated heated milk gel the particles were clearly connected or fused to each other, suggesting the presence of stronger inter-particle interactions. Such a difference between the particle interactions would explain the difference between the firmness of the two gels as detected by small and large deformation

measurements. However, it is challenging to explain the reasons for this finding. The following phenomena considering the reaction mechanisms of tyrosinase and transglutaminase were suggested:

1. Dopaquinones, created by oxidation of tyrosines, can bind with sulfhydryl groups in proteins (Kato et al., 1986). It is probable that in TrT-treated heated milk, free thiol groups were at least to some extent intra-molecularly coupled to tyrosine residues, thus partially limiting the formation of disulphide bonds. Disulphide linkages formed upon denaturation of β-lactoglobulin by pre-heat treatment greatly contribute to increased gel strength in heated milk (Vasbinder & de Kruif, 2003). Limitation of disulphide linkages might have counteracted the positive effect of tyrosinase-induced cross-links on gel firmness.

2. TG might preserve its activity for a longer duration during acidification as compared to TrT, thus contributing more to inter-micellar links. However, in acid-induced caseinate gels, both enzymes increased the gel firmness to highly comparable extents (see Figures 3 and 4 in Publication III), indicating that this might not be most relevant.

3. The reactivity of the micelles with each other by physical means is modified differently by TrT and TG. In heated milk, the micellar surface is modified by coupling of denatured whey proteins with κ-caseins (Guyomarc'h et al., 2003). Accordingly, differences in the extent of κ-casein or κ-casein/whey protein complex modifications at the micellar surface by the two enzymes might cause differences in inter-micellar reactivity.

It was shown that protein cross-linking (at the presented dosages) does not necessarily lead to increased gel firmness in acid milk gels. Cross-linking of micellar caseins by different enzymes influences the morphology of the final protein particles forming the gel network, and the interactions between these particles, in different ways in milk. However, in the caseinate system, in which the availability of caseins is different compared to the micellar state, both enzymes resulted in increased gel strength.

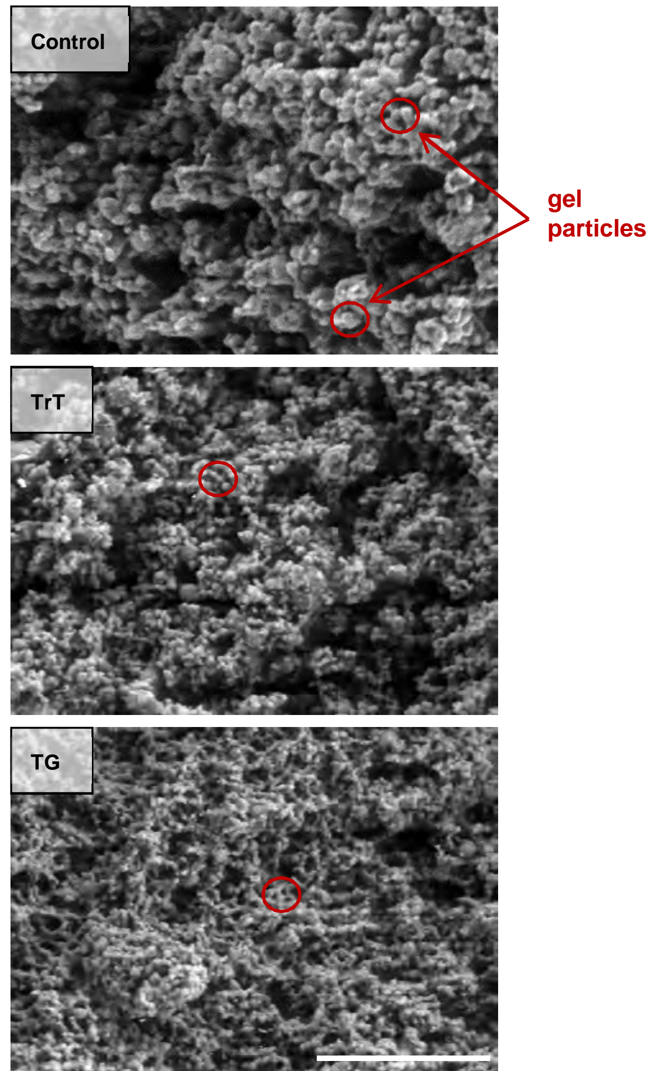

Figure 14. SEM images of heat-treated (90 °C, 5 min) milk gels. Before acidification, samples were treated with 100 nkat g^{-1} TrT or 25 nkat g^{-1} TG for 1 h at 40 °C. Acidification was performed at 30 °C, with 1.2% GDL. Scale bar represents 2 μm (Publication III).

4.2.3 Enzymatic cross-linking creates altered gel particles upon acidification of milk (Unpublished data and Publication IV)

As stated above, one significant outcome of imaging the enzyme-treated heated milk gels with SEM was the observed decrease in the size of the gel particles. The effect was also observed in TrT-treated raw milk gel, as shown in Figure 15 at high magnification. In order to observe the changes in particle/aggregate size during gelling, a light scattering technique was used which allows *in-situ* measurement of the original sample without any dilution. Turbiscan® measures the intensity of the backscattered light using a near-infrared light source. The intensity of the backscattered light (BS%) depends on the mean particle diameter, as well as the particle volume fraction and refractive indices. When the size of the particles is smaller than the wavelength of the incident light (880 nm), as in the case of milk, the increase in BS% corresponds to increasing particle/aggregate size. Accordingly, the lower values of BS% detected for the TrT-treated raw milk infer a smaller mean particle/aggregate size throughout the gelation (Figure 15B). In the lower graph of Figure 15B, the gelation curves as obtained by small deformation oscillatory measurements are also shown. They reveal the fact that the peaks seen in the BS% at 4–5 h for both samples coincide with the gelation point detected by rheology ($\tan\delta = 1$). After that point, the change in BS% was correlated directly with the particle-particle aggregate size (Castillo et al., 2006).

Near-infrared light-backscattering was also used to follow the gelation of heat-treated (80 °C, 30 min) milk gels treated by TG (see Figure 1 in Publication IV). Similarly, TG-treated samples reached lower BS% values all through the gelation which was performed at three different acidification temperatures; 20 °C, 30 °C and 40 °C. The lower intensity of the backscattered light indicated smaller aggregates, in agreement with the SEM images (Figure 14). In Publication IV, a higher TG dosage was used in order to intensify the effect of TG-induced cross-linking on casein micelles. Accordingly, diminution of the peak in $\tan\delta$ which occurred after the onset of gel formation (at acidification temperatures of 30 °C and 40 °C) was even more intensified compared to the lower TG dosage shown in Figure 13.

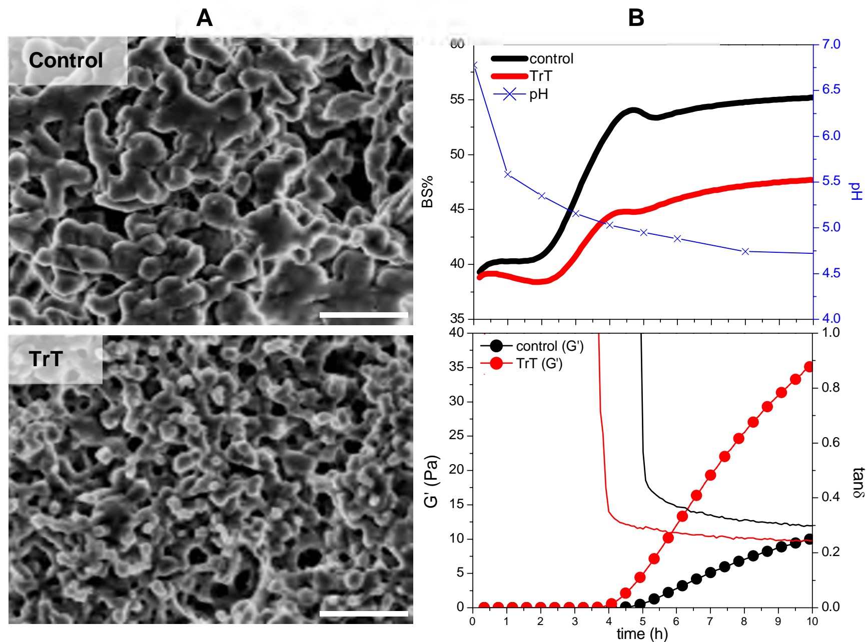

Figure 15. SEM images (A) of acid-induced milk gels prepared from raw milk without or with pre-treatment of TrT. Scale bar represents 500 nm. Gel formation (to pH 4.75) of the raw milk without or with pre-treatment with TrT (B) as followed by near-infrared light backscattering (upper graph) and small deformation oscillatory rheology (lower graph) (Unpublished data).

The observations above lead to the conclusion that pre-treatment of milk with cross-linking enzymes prior to acidification results in a gel composed of altered gel particles compared to a non-enzyme treated milk gel. This is suggested to be due to intra-micellar cross-links created by both TrT and TG which prevent the re-organization of the micellar caseins and casein dissociations (depending on the acidification temperature) that occur upon CCP removal (Figure 16). As a result, the particles forming the gel network show increased internal integrity and somehow constrained size and possibly have different aggregation dynamics compared to the native casein micelles.

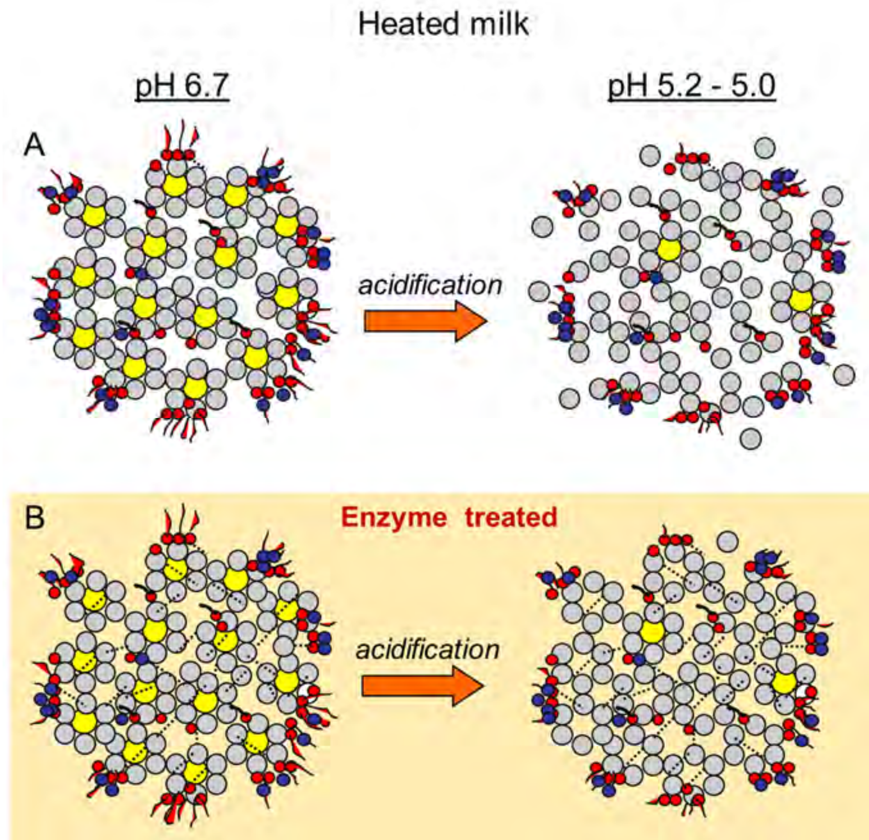

Figure 16. Schematic representation of the intra-micellar covalent bonds created by cross-linking enzymes and the effects on micelle structure upon acidification. When milk is acidified (A), intra-micellar interactions loosen upon solubilisation of CCP. When milk is pre-treated with cross-linking enzymes (B), micellar integrity is preserved upon CCP solubilisation. Color code: red: κ-caseins, dark blue: whey proteins; grey; α_s- and β-caseins; yellow: CCP; dotted lines represent enzyme-induced covalent linkages. Micelle structure was modified from Horne (2007). The sizes of the structural elements are not proportional to the real state.

4.2.4 The role of enzymatic cross-linking in improved water retention properties of acid-induced milk gels (Publication IV)

It was shown that acid gels made from enzyme-treated milk are composed of significantly smaller gel particles and aggregates organized in a lower mesh-sized network (Publication III). Acid milk gels from TG-treated milk have been reported to show less spontaneous syneresis and increased water holding capacity (WHC) (Lorenzen et al., 2002; Schorsch et al., 2000). Superior ability of enzyme-treated

acid milk gels to entrap water was studied by analysing the WHC and the microstructure of gels from TG-treated milk at certain pH points during gel formation. To even enhance such effects, a fourfold higher TG dosage was used compared to Publication III. Acidification rate, and thus aggregation dynamics, was changed by varying the acidification temperature (20 °C – 40 °C).

Both the acidification temperature and the TG treatment affected the WHC of the milk gels at different pH points significantly ($p < 0.05$) (Table 12). WHC increased with decreasing temperature at all pH points studied for both the control gels and the gels from TG-treated milk. TG treatment significantly increased the WHC values at all pH points for both acidification temperatures. The most remarkable result was that the WHC of TG-treated gels were superior to controls already at pH 5.0 and did not change until the final gels at pH 4.6 were formed. Spontaneous syneresis, which was observed in the 40 °C control gel, was prevented by TG treatment (Table 12).

Table 12. Water holding capacity (WHC) and the spontaneous syneresis values of the acid-induced milk gels. WHC results were collected at different pH points during acidification at 40 °C or 20 °C. The standard deviations are shown in brackets for each value. Statistical analysis was performed separately for the control gels (plain letters) and the gels from TG-treated skim milk (primed letters). Samples with different superscript letters within each group are significantly different (p<0.05). (SponS=Spontaneous syneresis) (Publication IV).

	WHC (%)				SponS (%)
	pH 5.2	**pH 5.0**	**pH 4.8**	**pH 4.6**	**pH 4.6**
40°C					
Control	28.4 (0.8) ^{a}	20.5 (0.2) ^{b}	18.2 (0.5) ^{c}	20.3 (0.4) ^{b}	12.9 (2.3)
TG	46.4 (1.0) $^{a'}$	57.5 (3.5) $^{b'c'}$	46.6 (1.8) $^{a'}$	53.7 (0.9) $^{b'}$	0.0
20°C					
Control	-	35.9 (0.5)* ^{d}	35.1 (0.4) ^{d}	37.5 (0.8) ^{e}	0.0
TG	-	78.6 (3.5)* $^{d'}$	81.3 (0.3) $^{d'}$	79.6 (0.9) $^{d'}$	0.0

*Water separation was not very clear

Proneness of a gel network to syneresis is related to the extent of network rearrangements during the gel formation, which subsequently leads to contraction of the network (van Vliet & Walstra, 1994). On the other hand, the ability of the gels to retain water under force (centrifugal force in this case) reflects their structural length scales due to hydrodynamic flow and capillary pressure as suggested by Hermansson (2008), thus directly related to the network structure, i.e. the pore size. At 40 °C, the control gel showed a changing microstructure from pH 5.2 to the final gel at pH 4.6, revealing large scale rearranging of the particle strand and clusters during gelation (Figure 17A). It attained a coarse structure with inhomogeneous pore size, which showed low WHC.

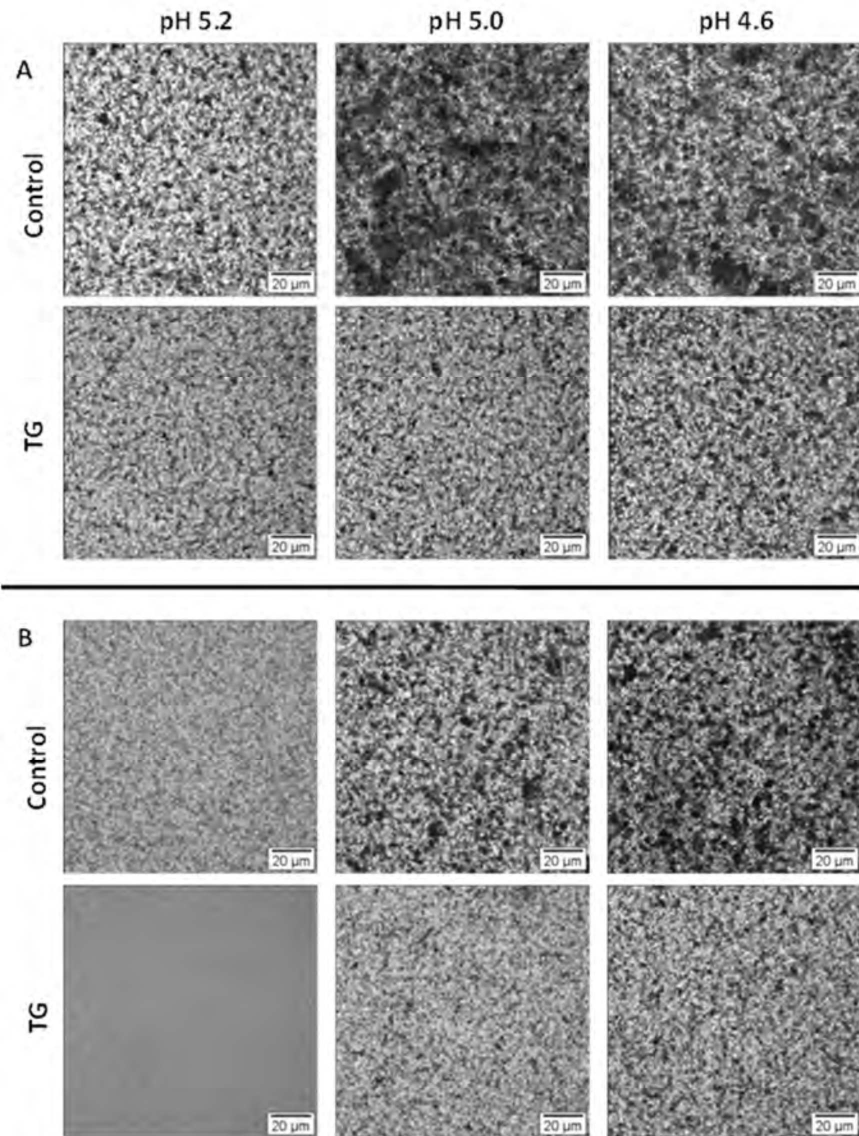

Figure 17. Confocal Scanner Laser Microscope (CLSM) images of the control gels and the gels from TG-treated skim milk at different pH points during acidification at 40 °C (A); and 20 °C (B) (Publication IV).

At 20 °C, large scale rearrangements of the network were limited, resulting in a more homogeneous and finer final network compared to the higher acidification temperature (Figure 17B). The relatively low extent of hydrophobic interactions and slower rate of pH drop at 20 °C prevent formation of large and inhomogeneous

clusters. The micrographs of the gels from TG-treated milk showed only minor changes from pH 5.2 to 4.6 at both acidification temperatures, revealing the formation of a low-mesh-sized, homogeneous protein network immediately after the gelation point, and showed only minor coarsening. The limited rearrangement potential of enzyme-treated gels was also indicated by the lack of peak in tanδ and overall lower tanδ values throughout the acidification. Moreover, smaller aggregate size was once more pronounced, and evidence that this changed the aggregation dynamics was obtained.

The results indicate the importance of the initial state of the gel particles and particle-particle interactions, i.e. aggregation dynamics and rearrangement potential of the system, in determining the water holding properties and proneness of a gel network to spontaneous syneresis. This phenomenon is depicted in Figure 18. The particle size differences between the control and the TG–treated gels were exaggerated for emphasis. Although the effect of intra-micellar cross-linking on the size of the particles around the gelation point was not studied directly, the indirect evidence obtained by near-infrared light backscattering measurements and the micrographs (both SEM and CLSM) were taken into consideration.

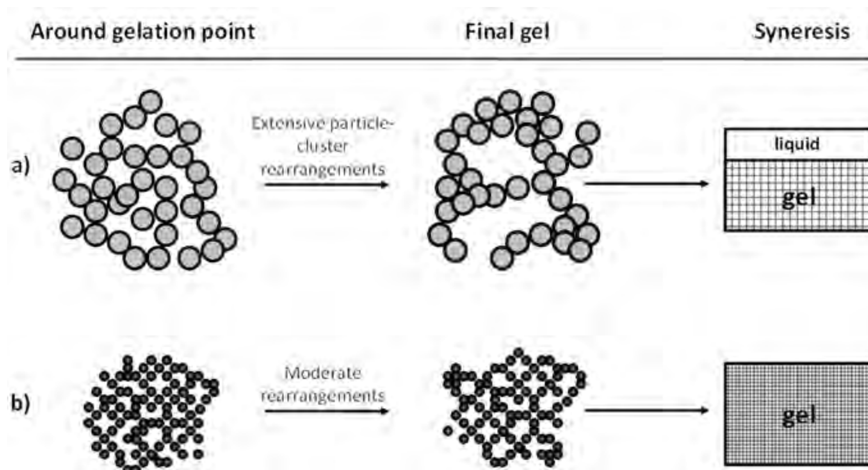

Figure 18. Formation and the final structure of milk gels without (a) and with (b) transglutaminase.

5. Summary and conclusions

The potential of enzymatic protein cross-linking in modification of acid-induced milk protein gel structures was studied by using the oxidative enzymes laccase and tyrosinase as well as the acyltransferase transglutaminase. The efficiency of different cross-linking enzymes with dissimilar reaction mechanisms in modification of milk proteins at various colloidal (in milk or in caseinate) or molecular (native, unfolded) states was studied. The effects of enzyme-induced inter-molecular covalent linkages on structural attributes of acid-induced milk protein gels were investigated. Finally, the mechanisms leading to improved water holding properties in enzyme-treated milk gels were elucidated.

In the first part of the study, the efficiency of the enzymes in cross-linking of milk proteins in skim milk, caseinate or whey protein solutions was assessed. Laccase was not able to induce inter-molecular protein cross-linking unless used in high dosage. The cross-linking efficiency of laccase on caseins was increased by the presence of FA. Laccase also fragmented the proteins, which was mainly attributed to the minor protease contamination detected in the enzyme preparation, but was also considered to be caused by the radical-induced reaction mechanism of laccase. Two tyrosinases; a commercially available *A. bisporus hirsuta* tyrosinase, and a *T. reesei* tyrosinase produced and characterized at VTT, were used. In heat treated milk, TrT and TG formed inter-molecular linkages, most efficiently between β-caseins. In raw milk, only TrT was able to form inter-molecular covalent links. TrT was shown to be a superior enzyme compared to AbT in terms of milk protein cross-linking. Cross-linking of whey proteins was extensively studied by using purified β-lactoglobulin. BLG molecule is not susceptible to enzymatic reactions unless the globular structure is partially unfolded. Thus, an attempt was made to link the changes in secondary and tertiary structures of BLG to TrT- and TG-catalysed reactions. At pH 7.5 and 9.0, although only minor shift in the near-UV spectra of BLG was detected (only at pH 9.0), TG was able to induce cross-linking whereas TrT did not. A substantial change in both near and far-UV ellipticities was needed for more efficient cross-linking of BLG with both enzymes. The roles of other physicochemical changes induced by pH and heat treatments on enzymatic cross-linking were also considered. Better efficiency of TG compared to TrT was attributed to the higher number and better accessibility of glutamine and lysine residues compared to tyrosines in the BLG molecule. TG-induced oligomer bands of BLG showed increased mobility on SDS-PAGE gels compared to those induced

by TrT. This was attributed to a change in shape (compactness) of the TG-induced oligomers. Modification of adsorbed layers of BLG at air-water interface was also studied and evidence for extensive intra-molecular cross-linking with subsequent limitation of physical interactions between BLG molecules was obtained. By using different enzymes with different modes of action, protein interactions and thus product attributes might be tuned differently.

In the second part of the study, structure modification of acid-induced milk protein gels by enzymatic cross-linking was assessed. Laccase, when used together with FA, increased the firmness of the caseinate gels and induced formation of a finer microstructure. Moreover, FA was shown to be incorporated into the casein polymers formed by laccase-induced cross-linking. Accordingly, grafting of phenolic substances by use of laccase for increased nutritional value or antioxidant properties in dairy products might have future potential. Laccase as such was not found to be an effective structure improver in acid-induced milk protein gels. In a separate study, the effects of tyrosinase and TG treatments on the mechanical properties and microstructure of acid milk and caseinate gels were compared. Inter-molecular links attained by different cross-linking enzymes led to distinctive gel structures in milk but not in caseinate. The results were discussed regarding the colloidal nature of casein micelles and their interactions. In raw milk, TrT was the only enzyme able to increase the gel firmness as analysed by both small and large deformation measurements. This makes TrT a potential enzyme for use in raw milk-based products such as cheese. In heated milk, on the other hand, even though TrT could induce casein cross-linking to a similar extent as TG, no impact on final G' or firmness measured by large deformation measurements was detected. However, both enzymes induced the diminution of peak in tanδ, which was observed in the control sample immediately after the gelation point. Obviously, TrT treatment altered the intra-micellar interactions in casein micelles similarly to TG, giving them increased stability against solubilisation of CCP compared to the non-enzyme treated micelles. SEM micrographs revealed that pre-treatment of milk by TrT or TG led to substantially smaller gel particles upon acidification compared to the untreated control gels. The main difference between the TrT- and TG-treated gels was observed to be in the inter-particle interactions. Obviously, reactivity of the casein micelles was affected differently by cross-links attained with different enzymes in heated milk. The results were also significant in terms of showing TrT to create altered gel particles both in raw and heated milk gels.

In the last part of the study, formation dynamics and the mechanism behind the improved water holding properties of acid milk gels prepared from TG-treated milk were elucidated. It was verified that enzyme-treated gel was fixed into a fine network structure immediately after the gelation point and did not show any large-scale rearrangements thereafter. Small-sized aggregates and decreased rate of network organization led to a gel arranged in a low-mesh sized network which entraps water efficiently throughout gel formation.

The results presented in this study have shown that enzymatic cross-linking, even with the non-conventional enzymes tyrosinase and laccase, alters the mechanical properties of acid-induced milk protein gels. However, the knowledge

on the mode of action of these enzymes on proteins should be further elucidated in order to be able to exploit them as structure-engineering tools with maximum value. It was found that it is not solely the introduced covalent links but also the preceding impacts on colloidal interactions by physical means (e.g. hydrophobic interactions) which determine the actual effect of cross-linking on the final product attributes. Comparison of tyrosinase and transglutaminase directly in milk, in which caseins are found as association colloids, showed that even rather similar intra-particle covalent linkages did not necessarily result in similar mechanical properties in final acid-induced gels. At this point, identification of the micellar proteins that are cross-linked differently by TG and TrT would help us to understand the actual reasons behind the altered inter-particle interactions. For example, the extent of cross-linking by tyrosinase in κ-casein and micelle-bound κ-casein/whey protein complexes on the micellar surface should be verified and compared to that caused by TG. In this thesis, the potential of one oxidative enzyme, *T. reesei* tyrosinase, was demonstrated for the creation of stable casein particles by intra-micellar cross-linking. In the future, it will be necessary to determine the physicochemical properties of TrT-induced casein particles and their industrial value as compared to the TG-induced casein particles. Finally, elucidation of altered aggregation dynamics for cross-linked protein particles will help to determine the optimum production parameters in order to tailor protein gels for improved product characteristics.

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