

Structure modification of milk protein gels by enzymatic cross-linking

Dilek Ercili-Cura



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VTT

PL 1000 (Tekniikantie 4 A, Espoo)

02044 VTT

Puh. 020 722 111, faksi 020 722 7001

VTT

PB 1000 (Teknikvägen 4 A, Esbo)

FI-02044 VTT

Tfn +358 20 722 111, telefax +358 20 722 7001

VTT Technical Research Centre of Finland

P.O. Box 1000 (Tekniikantie 4 A, Espoo)

FI-02044 VTT, Finland

Tel. +358 20 722 111, fax + 358 20 722 7001

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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 $\tan\delta$ 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 $\tan\delta$, 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 ($>30\text{ }^{\circ}\text{C}$), 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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I warmly thank to Prof. Kristiina Kruus, Dr. Harry Boer, Doc. Maija-Liisa Mattinen, Dr. Pirkko Forssell, Dr. Emilia Nordlund who were involved in the Pro-Enz and never withheld their valuable comments and suggestions on my work.

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Espoo, October 2012,
Dilek

Academic dissertation

- Supervisors Dr. Raija Lantto
VTT, Technical Research Center of Finland
Espoo, Finland
- Dr. Riitta Partanen
VTT, Technical Research Center of Finland
Espoo, Finland
- Prof. Johanna Buchert
VTT, Technical Research Center of Finland
Espoo, Finland
- Reviewers Dr. Päivi Myllärinen
Valio Oy
Helsinki, Finland
- Dr. Thom Huppertz,
NIZO food research
EDE, The Netherlands
- Opponent Prof. Alan Kelly
School of Food and Nutritional Sciences
University College Cork, Cork, Ireland
- Custos Prof. Tapani Alatossava
Department of Food and Environmental Sciences,
University of Helsinki, Helsinki, Finland

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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List of symbols

AbT	<i>Agaricus bisporus</i> 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	Caseinomacropptide
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	<i>Trametes hirsuta</i> laccase
L-DOPA	L-Dihydroxyphenylalanine
LVR	Linear viscoelastic region

MW	Molecular weight
nkat	Nanokatal
<i>p</i> -CA	<i>para</i> -coumaric acid
pI	Isoelectric point
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SEM	Scanning Electron Microscopy
tan δ	Loss tangent
TG	<i>Streptomyces mobaraensis</i> transglutaminase
TrT	<i>Trichoderma reesei</i> 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.

Table 1. 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

1. Introduction

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 & Brodtkorb, 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

^aVariant B, ^bVariant A, ^cVariant A², ^dExclusive of carbohydrates ^eOccasionally 9, ^fOccasionally 4,

^gOccasionally 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 phosphoserine residues, making it soluble in the presence of Ca^{2+} 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 κ -casein, called caseinomacropptide (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).

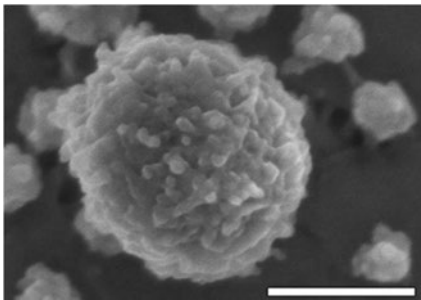


Figure 1. SEM image of a casein micelle. The bar represents 200 nm. Reprinted from (Dalgleish et al., 2004), copyright with permission from Elsevier.

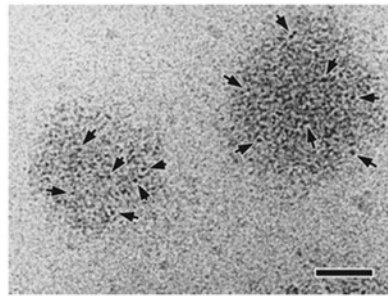


Figure 2. Cryo-TEM image of casein micelles. The bar represents 50 nm. Reprinted from (Marchin et al., 2007), copyright with permission from American Institute of Physics.

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
	<ul style="list-style-type: none"> 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. 	<ul style="list-style-type: none"> 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)
(Schmidt, 1980; Walstra, 1990)		
	<ul style="list-style-type: none"> Phosphoserine 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 phosphoserine clusters, they connect the nanoclusters to each other forming a growing network with a uniform distribution of nanoclusters. 	<ul style="list-style-type: none"> 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).
(Holt, 1992; Holt et al., 1998)		
	<ul style="list-style-type: none"> α_s- 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. 	<ul style="list-style-type: none"> Describes the intra-micellar interactions but does not 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)
(Horne, 1998; Horne, 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 lipocalin 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 ($>70^{\circ}\text{C}$) 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 ($\sim\text{pH } 5.4$ 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 ($>40^\circ\text{C}$) 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.

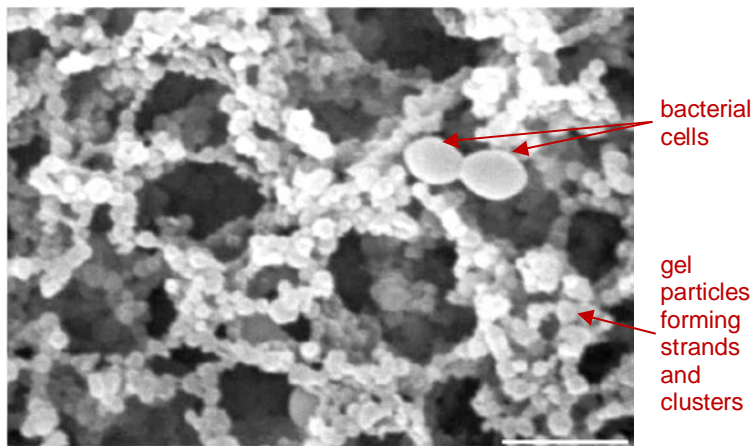


Figure 3. SEM image of yoghurt fermented with lactic acid bacteria. Casein particles form clusters and strands entrapping the water phase in the pores. Two bacterial cells are also visible. The scale bar represents 2 μm . Modified from Kalab (2010).

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 $\tan\delta$ value. Accordingly, a high $\tan\delta$ during the initial phases of gel formation or high final $\tan\delta$ 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 -carboxamide 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).

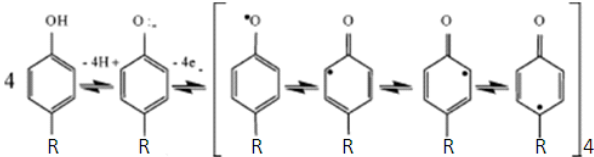
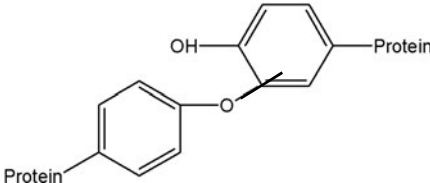
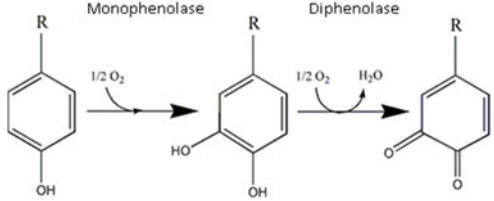
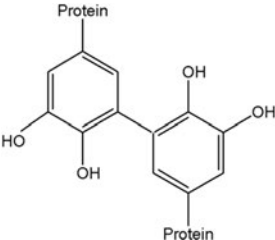
Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are copper-containing oxidases which generate free radicals and utilize molecular oxygen as an electron acceptor. The physiological functions of laccases are mostly related to polymerization or degradation reactions of lignin (Gianfreda et al., 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 sulphhydryl 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 proteinaceous 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	$\begin{array}{c} \\ \text{Gln} - \text{C} - \text{NH}_2 \\ \quad \quad \quad \\ \quad \quad \quad \text{O} \end{array} + \text{H}_2\text{N} - \text{Lys}$	$\text{Protein} - (\text{CH}_2)_2 - \text{C}(=\text{O}) - \text{NH} - (\text{CH}_2)_4 - \text{Protein}$
Laccase ^{b,c}	 <p>The diagram illustrates the redox cycle of laccase. It starts with 4 molecules of a phenol (R-C₆H₄-OH). The first step is the oxidation of the phenol to a semiquinone radical (R-C₆H₄-O[•]), releasing 4H⁺ and 4e⁻. The semiquinone radical then undergoes further oxidation to a quinone (R-C₆H₄-O=O), which is then reduced back to the semiquinone radical, and so on, forming a cycle of four semiquinone radicals.</p>	 <p>The diagram shows a cross-link formed by laccase. Two protein-bound phenols (Protein-C₆H₄-OH) are linked together by an ether bridge (C₆H₄-O-C₆H₄-Protein).</p>
Tyrosinase ^d	 <p>The diagram shows the reaction scheme for tyrosinase. A monophenol (R-C₆H₄-OH) is first converted to a diphenol (R-C₆H₃(OH)₂) by the action of Monophenolase, releasing 1/2 O₂. The diphenol is then converted to a catechol (R-C₆H₂(OH)₂) by the action of Diphenolase, releasing 1/2 O₂ and H₂O.</p>	 <p>The diagram shows a cross-link formed by tyrosinase. Two protein-bound catechols (Protein-C₆H₂(OH)₂) are linked together by a biaryl bridge (C₆H₂(OH)₂-C₆H₂(OH)₂-Protein).</p>

^amodified from (Kuraishi et al., 2001), ^bmodified from (Mattinen et al., 2005), ^cValidated for high redox-potential laccases, ^dmodified 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 Kruijff, 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 Kruijff, 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 <i>T.reesei</i> tyrosinase cross-linked caseins	Mattinen et al. (2008b)
	α_s -caseins	L-dopa (negative effect)	Almost complete oligomerization obtained with <i>T.reesei</i> tyrosinase	Selinheimo et al. (2007b)
Tyrosinase & laccase	α_s -caseins	FA & <i>p</i> -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 <i>T.reesei</i> 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).

1. Introduction

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

Gel preparation	Effects on final gel	References
<ul style="list-style-type: none"> Heat-treated milk (92°C, 5 min) was incubated with TG. TG was either inactivated or not prior to acidification. Starter bacteria, 43°C. 	<ul style="list-style-type: none"> 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)
<ul style="list-style-type: none"> Pasteurized milk treated with TG under high pressure. TG was inactivated prior to acidification. GDL, 30°C. 	<ul style="list-style-type: none"> 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)
<ul style="list-style-type: none"> Raw skim milk was incubated with TG. TG was inactivated prior to acidification. Starter bacteria, 42°C. 	<ul style="list-style-type: none"> 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)
<ul style="list-style-type: none"> Heat-treated milk (90°C, 5 min) was incubated with TG. TG was either inactivated or not prior to acidification. Starter bacteria, 43°C. 	<ul style="list-style-type: none"> 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)
<ul style="list-style-type: none"> UHT milk was incubated with TG. TG was either inactivated or not prior to acidification. GDL, 30°C. 	<ul style="list-style-type: none"> 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)
<ul style="list-style-type: none"> 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). 	<ul style="list-style-type: none"> 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
<ul style="list-style-type: none"> • Sodium caseinate solution (2.7 and 4.5%). • TG was added simultaneously with GDL. • Acidification at 4, 22, 37 and 50°C. 	<ul style="list-style-type: none"> • Increased gel firmness with TG at all acidification temperatures. • Finer and more homogeneous microstructure in TG-treated gels. 	Myllärinen et al. (2007)
<ul style="list-style-type: none"> • Acid casein solution (5%) was incubated with TG. • Acidification at 40°C, at varying GDL dosages. 	<ul style="list-style-type: none"> • 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)
<ul style="list-style-type: none"> • 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. 	<ul style="list-style-type: none"> • 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).

3. Materials and methods

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

	Information	Publication
Enzymes		
Laccase	Origin: <i>T. hirsuta</i> . Produced and purified at VTT ^a	II
Tyrosinase	Origin: <i>T. reesei</i> . Produced and purified at VTT ^b	I, III
	Origin: <i>A. bisporus</i> . Commercial preparation (Fluka Biochemica, Taufkirchen, Germany)	
Transglutaminase	Origin: <i>S. mobaraensis</i> . 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-glutaminyglycine	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: ^aRittstieg et al., 2002, ^bSelinheimo et al., 2006, ^cLantto et al., 2005, ^dNiku-Paavola et al., 1988, ^eRobb, 1984, ^fFolk, 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 tempering 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	100	1 h at 40°C	30°C
	Milk	TG	25		
IV	Reconstituted milk	TG	100	1 h at 40°C	20°C, 30°C, 40°C

^a with/without 2.5 mM FA, ^bBased 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.

3. Materials and methods

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 $\tan\delta$ 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, $\delta = 0^\circ$ (in-phase), for a pure liquid $\delta = 90^\circ$ (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:

$$\sigma = \sigma_0 \sin(\omega t + \delta) \quad (3.1)$$

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

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

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

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

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

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

$$\sigma = G'(\omega)\gamma_0 \sin \omega t + G''(\omega)\gamma_0 \cos \omega t \quad (3.5)$$

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 $\tan \delta$ which corresponds to:

$$\tan \delta = \frac{G''}{G'} \quad (3.6)$$

In a gelling system, the point of gel formation can be defined as the point at which $\tan \delta = 1$ ($G' = G''$) (Horne, 1999). After that point $\tan \delta$ 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).

3. Materials and methods

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 (\varnothing 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).

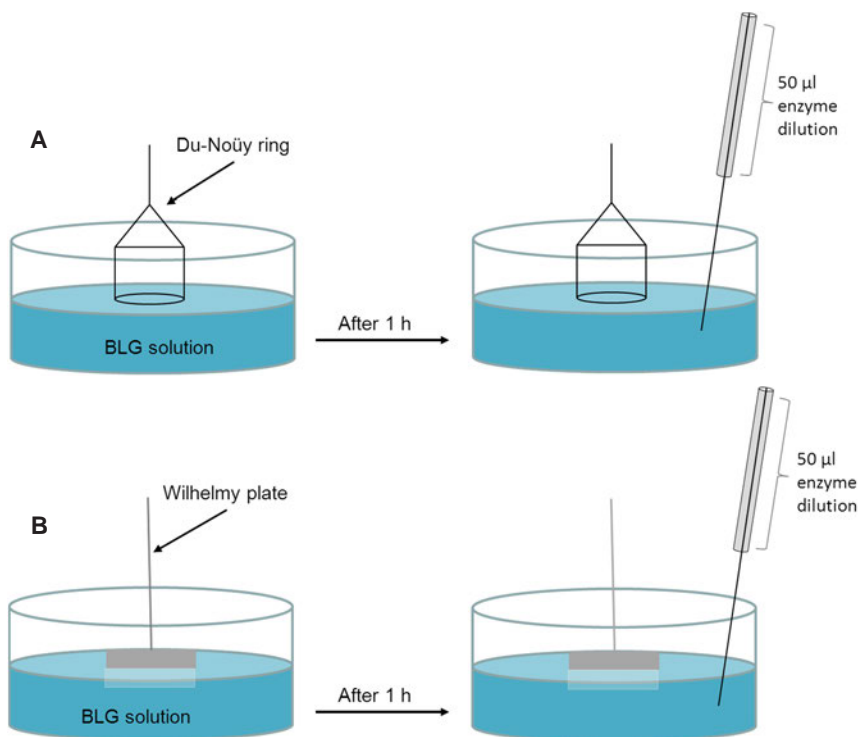


Figure 4. Representation of the interfacial shear rheology (A) and surface pressure (B) measurements which were performed simultaneously (Publication I).

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).

4. Results and discussion

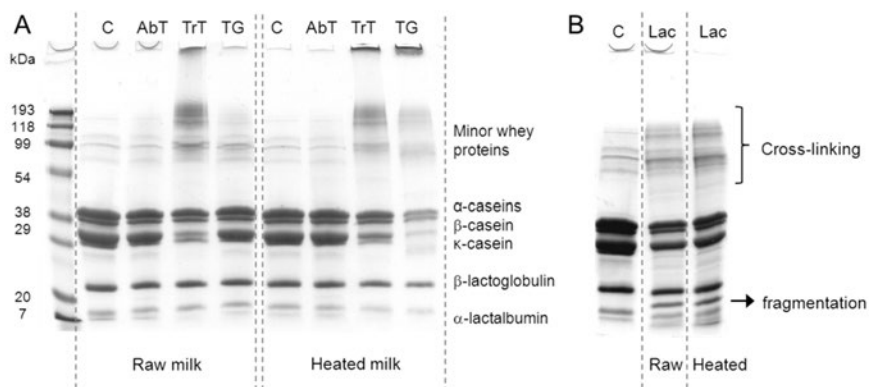


Figure 5. SDS-PAGE analysis of enzyme-treated raw or heated (90°C, 5 min) milk. The milk was incubated with AbT, TrT, TG (A) and Lac (B) for 1 h at 40 °C. The enzyme dosage was 500 nkat g⁻¹ of protein for all enzymes (unpublished data).

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.

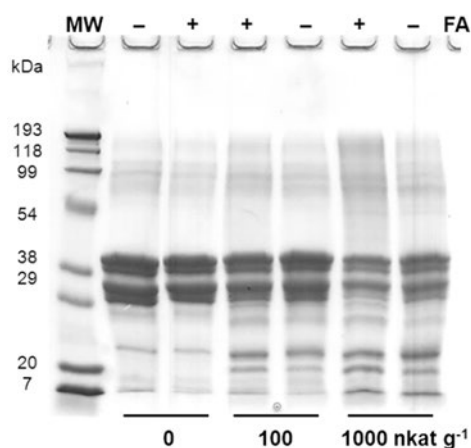


Figure 6. Cross-linking of 0.3% sodium caseinate solution (in 0.1M NaP buffer at pH 7.0) with *T. hirsuta* laccase (Lac) with or without 2.5 mM FA at room temperature for 2 h. (Enzyme activity based on oxygen consumption on 10 mM FA at pH 7.0) (unpublished data).

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

4. Results and discussion

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.

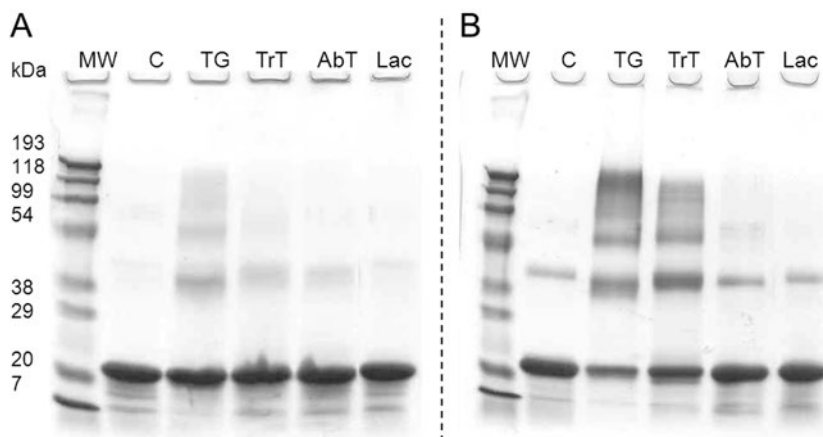


Figure 7. Cross-linking of β -lactoglobulin (1.7 mg mL^{-1} in 50 mM sodium phosphate buffer at pH: 8.0) at $40 \text{ }^\circ\text{C}$ for 20 h without any heat treatment (A) and after heat-treatment at $80 \text{ }^\circ\text{C}$, 15 min (B). The enzyme dosage was 1000 nkat g^{-1} of protein for all 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).

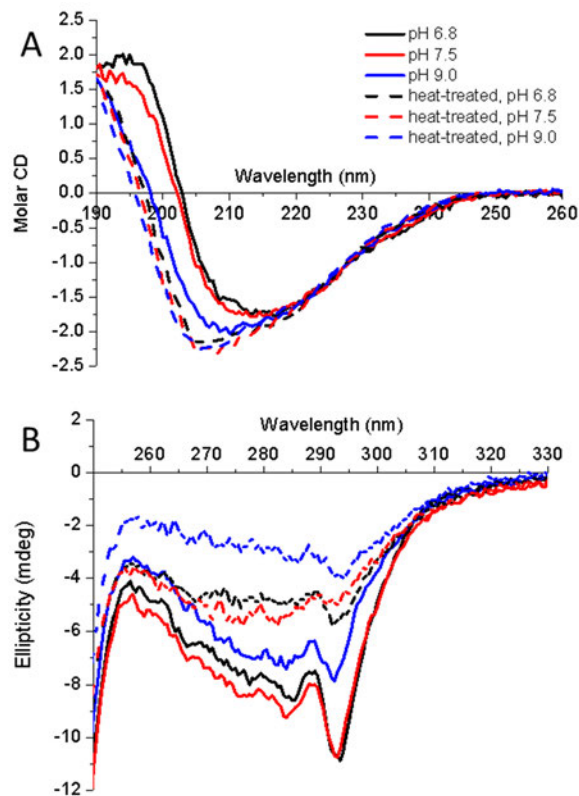


Figure 8. Far-UV (A) and Near-UV (B) CD spectra of non-heated (solid lines) and heated (80°C, 30 min) (dashed lines) BLG molecules at pH 6.8 (black), pH 7.5 (red), and pH 9.0 (blue) (Publication I).

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; Townsend et al., 1969). Thus, the number of accessible tyrosine residues might still be highly limited without further unfolding.

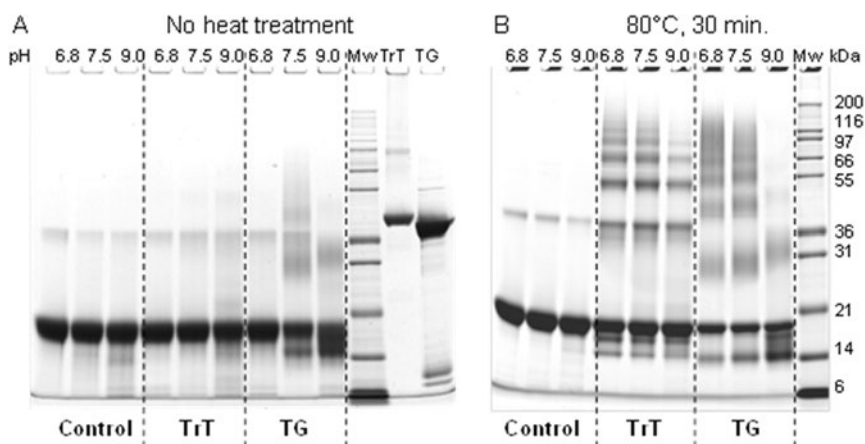


Figure 9. Reducing SDS-PAGE of BLG at pH 6.8, 7.5 and 9.0 without any heat-treatment (A) and after heat treatment at 80°C, 30 min. (B). BLG was cross-linked by 1000 nkat g⁻¹ TrT and 1000 nkat g⁻¹ TG for 18 h at RT and 40°C, respectively. The last two lanes (A) represent the enzymes loaded alone (Publication 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 $\sim 20 \text{ mN m}^{-1}$ 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.

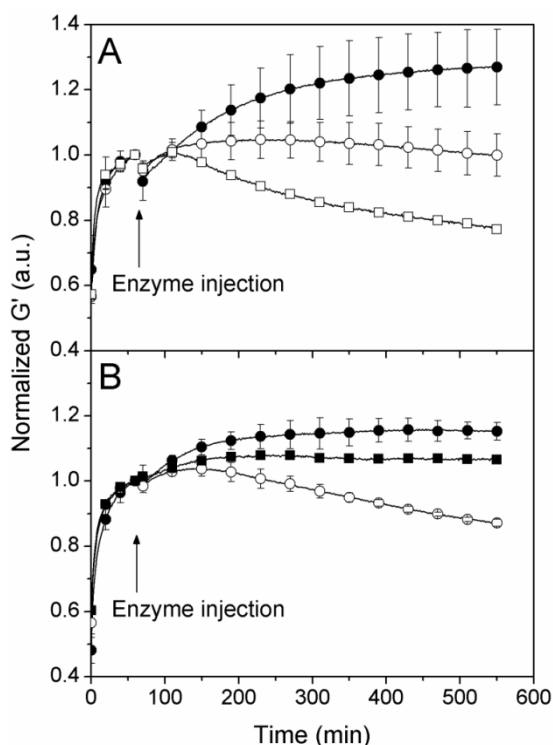


Figure 10. Effect of TG on interfacial shear elastic modulus (G') of non-heated (A); and heated (80°C , 30 min) (B) BLG solutions at pH 6.8. BLG adsorption was followed for 1h, after which $10\,000\text{ nkat g}^{-1}$ TG was injected to the sub-phase (\circ). Only buffer was injected to the control samples (\bullet). In addition, a curve with $50\,000\text{ nkat g}^{-1}$ TG injection (\square) is shown in (A), and a curve with inactivated TG injection (\blacksquare) 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'; in fact, 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).

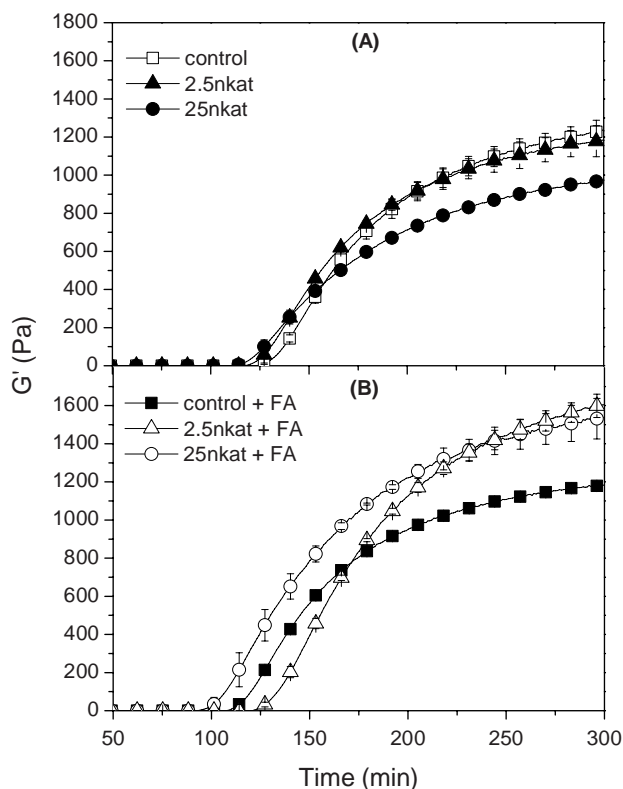


Figure 11. Effect of laccase on the storage modulus (G') of caseinate solution during acidification. Before acidification (with 1.13% GDL at 25 °C), sodium caseinate solutions were pre-treated for 2 h at 45 °C by: Laccase (A) or laccase + 2.5 mM FA (B) with the dosages; control without laccase and FA (\square); control + FA (\blacksquare); 2.5 nkat g^{-1} laccase (\blacktriangle); 25 nkat g^{-1} laccase (\bullet); 2.5 nkat g^{-1} laccase + FA (\triangle); 25 nkat g^{-1} laccase + FA (\circ). 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.

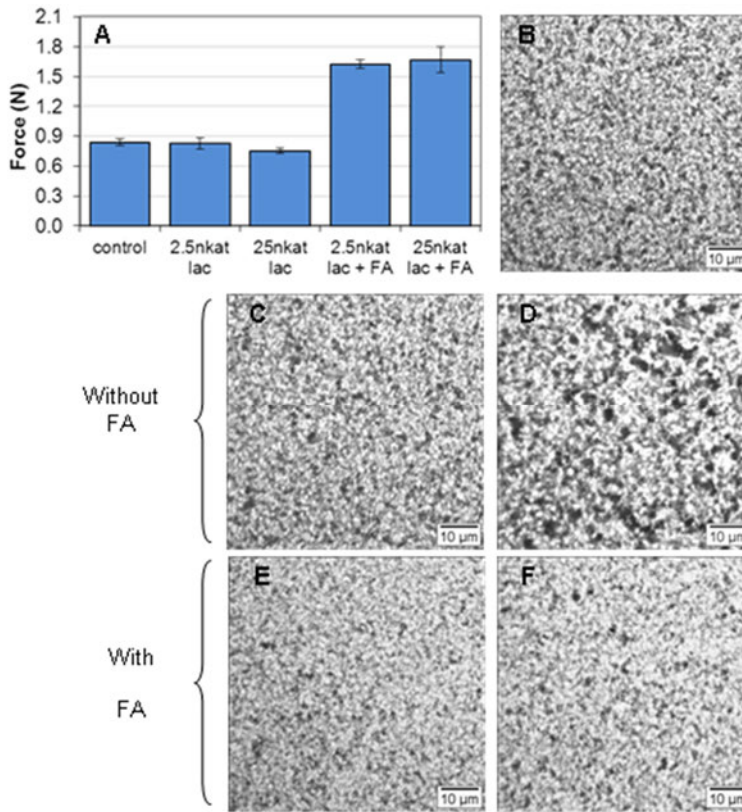


Figure 12. Firmness (force at rupture point) graph (A) and the CLSM images of sodium caseinate gels acidified at 25 °C with 1.13% GDL. Before acidification, sodium caseinate solutions were pre-treated by laccase for 2 h at 45 °C with the dosages; control without laccase and FA (B); 2.5 nkat g⁻¹ laccase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

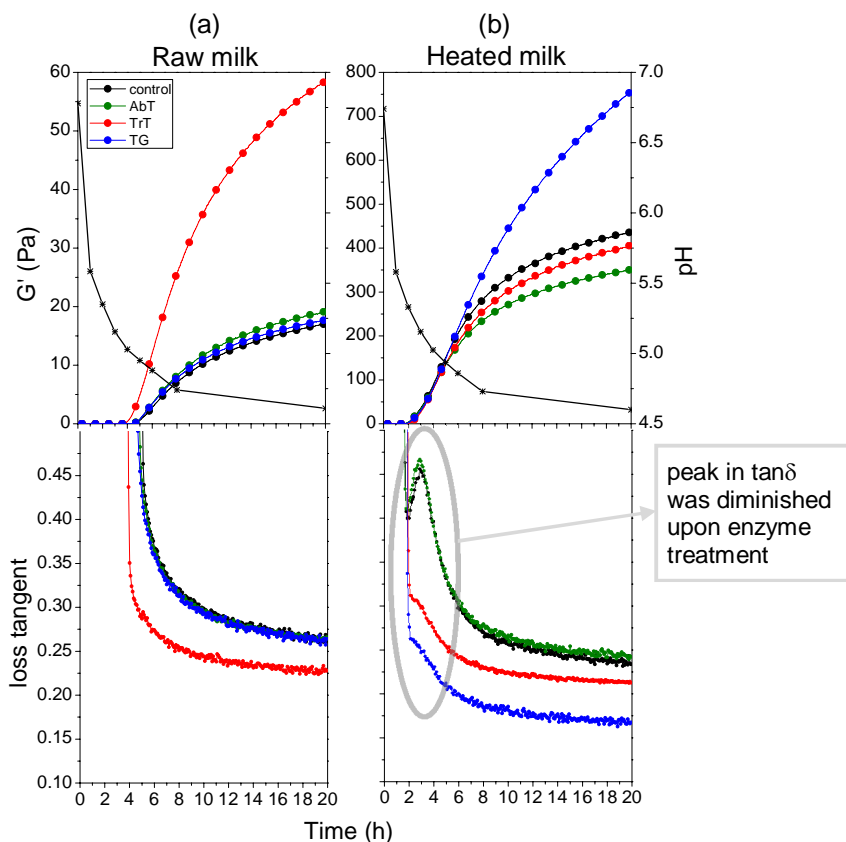


Figure 13. Storage modulus (G') and loss tangent of acidified raw milk (a) and heated (90 °C, 5 min) milk (b) gels. Before acidification, samples were treated by 100 nkat g^{-1} AbT, 100 nkat g^{-1} TrT, or 25 nkat g^{-1} TG, for 1 h at 40 °C. Acidification was performed at 30 °C, with 1.2% GDL. The 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 $\tan\delta$ 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 $\tan\delta$ 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 $\tan\delta$ 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 $\tan\delta$ values during acidification. Furthermore, the local maximum (peak) in $\tan\delta$ 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 $\tan\delta$ 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 $\tan\delta$ 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

4. Results and discussion

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. Dopaoquinones, 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.

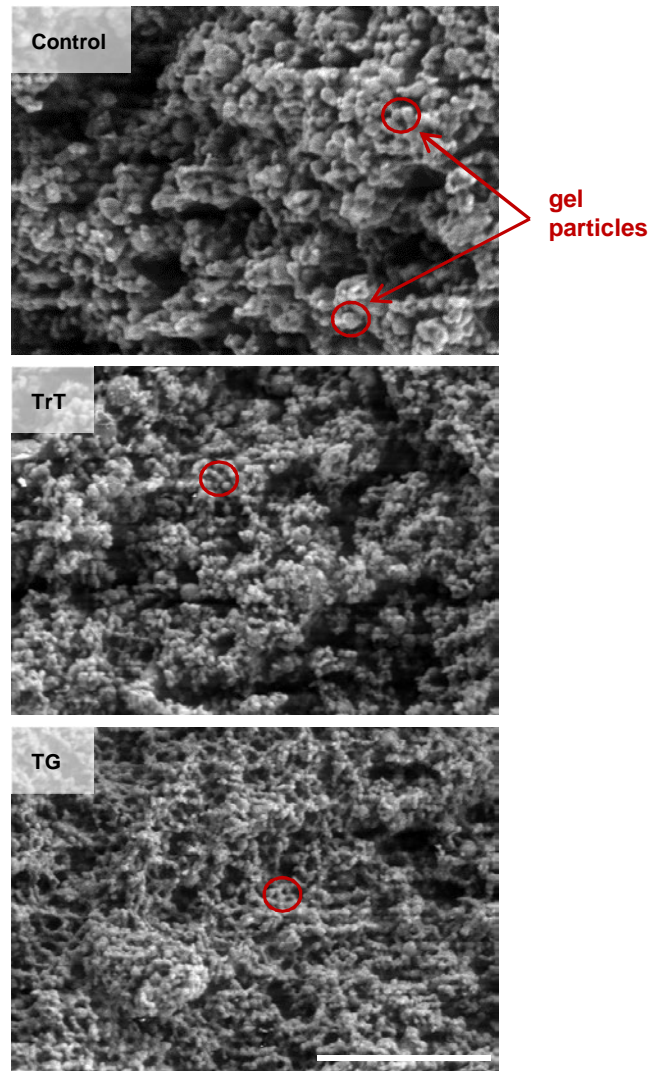


Figure 14. SEM images of heat-treated (90 °C, 5 min) milk gels. Before acidification, samples were treated with 100 nkat g⁻¹ TrT or 25 nkat g⁻¹ TG for 1 h at 40 °C. Acidification was performed at 30 °C, with 1.2% GDL. Scale bar represents 2 μm (Publication III).

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.

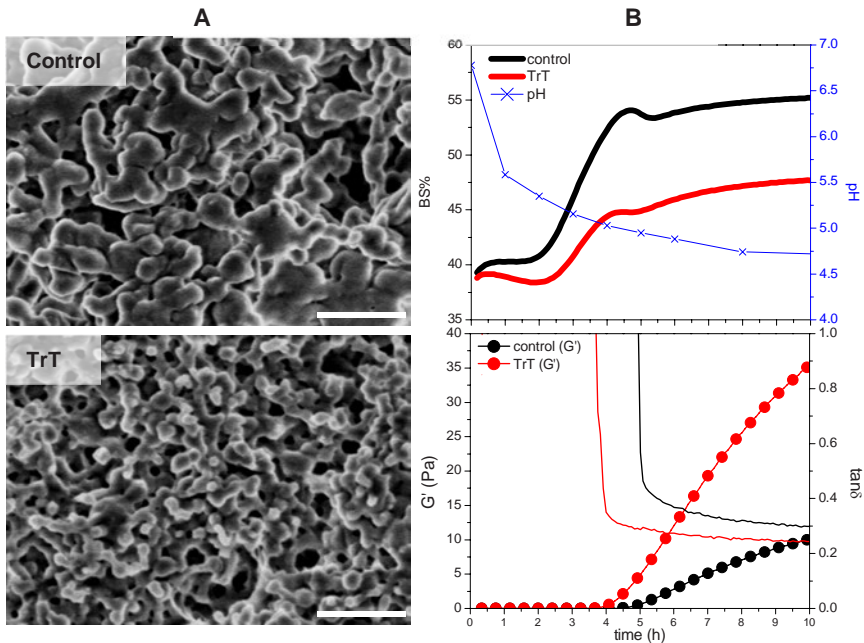


Figure 15. SEM images (A) of acid-induced milk gels prepared from raw milk without or with pre-treatment of TrT. Scale bar represents 500 nm. Gel formation (to pH 4.75) of the raw milk without or with pre-treatment with TrT (B) as followed by near-infrared light backscattering (upper graph) and small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

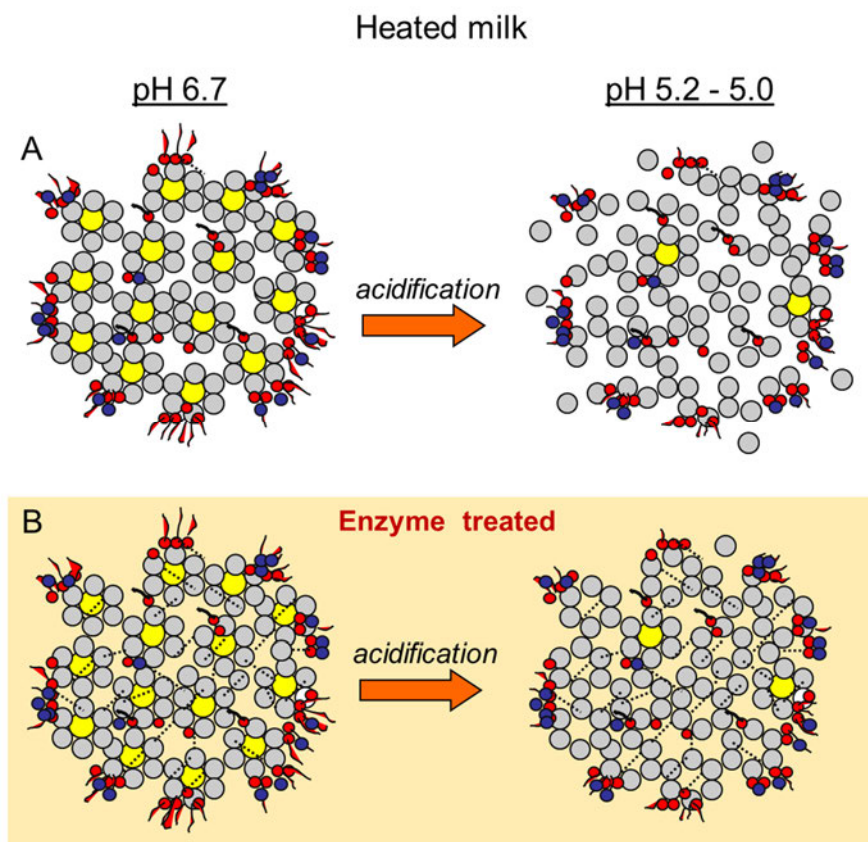


Figure 16. Schematic representation of the intra-micellar covalent bonds created by cross-linking enzymes and the effects on micelle structure upon acidification. When milk is acidified (A), intra-micellar interactions loosen upon solubilisation of CCP. When milk is pre-treated with cross-linking enzymes (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 Home (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.

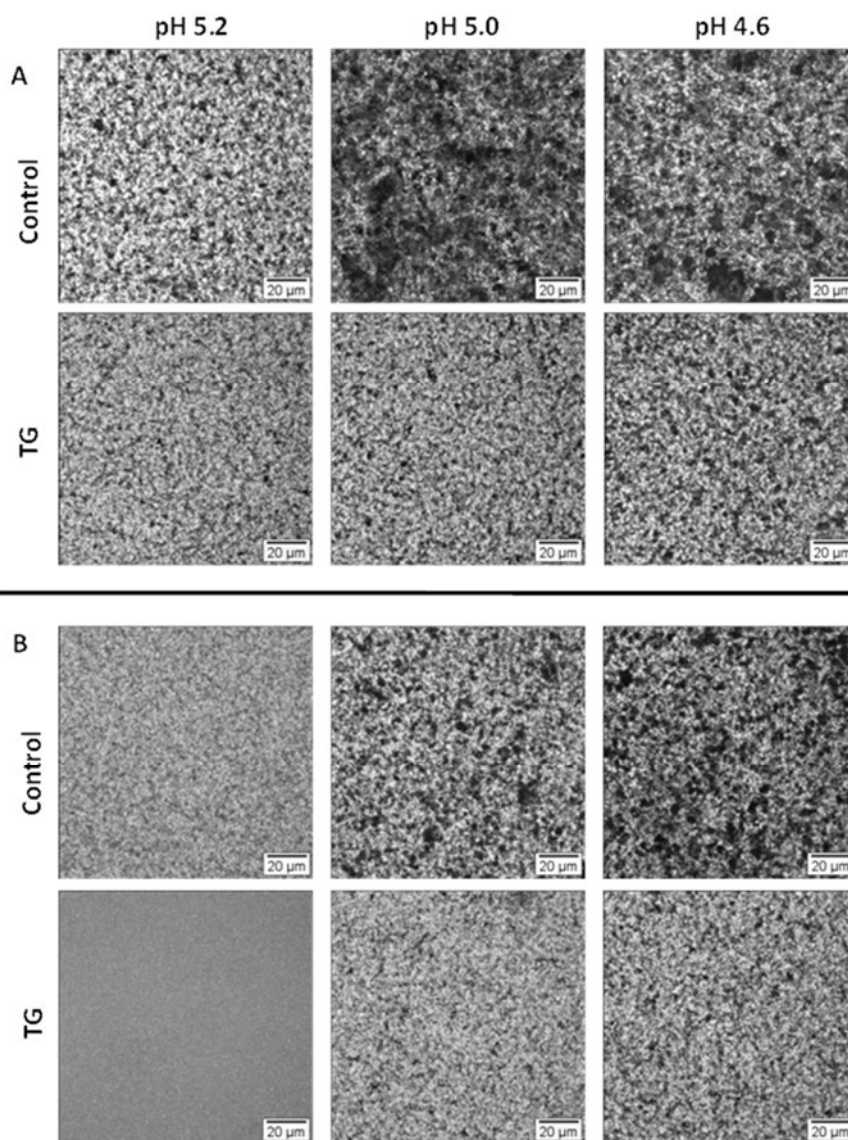


Figure 17. Confocal Scanner Laser Microscope (CLSM) images of the control gels and the gels from TG-treated skim milk at different pH points during acidification at 40 °C (A); and 20 °C (B) (Publication IV).

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 $\tan\delta$ and overall lower $\tan\delta$ 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.

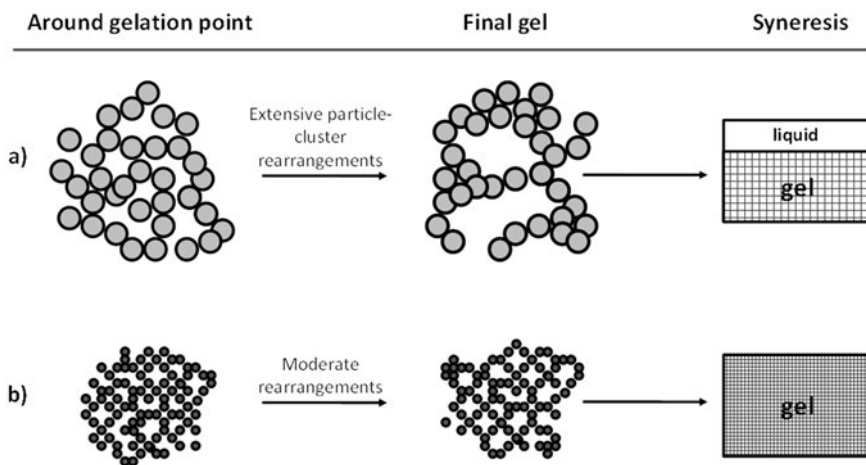


Figure 18. Formation and the final structure of milk gels without (a) and with (b) transglutaminase.

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 $\tan\delta$, 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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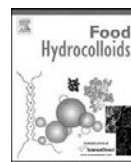
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PUBLICATION I

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 β -lactoglobulin in solution and
at air-water interface
Structural constraints**

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Enzymatic cross-linking of β -lactoglobulin in solution and at air–water interface: Structural constraints

Dilek Ercili-Cura^{a,*}, Riitta Partanen^a, Fiona Husband^b, Mike Ridout^b, Adam Macierzanka^b, Martina Lille^a, Harry Boer^a, Raija Lantto^a, Johanna Buchert^a, Alan R. Mackie^b

^aVTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Espoo, Finland

^bInstitute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK

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ABSTRACT

Effective and controlled use of cross-linking enzymes in structure engineering of food systems depends on characterization of the favorable conditions for enzyme-substrate complex and the limiting factors for the desired modification. In this respect, we analyzed the susceptibility of bovine β -lactoglobulin (BLG) to enzymatic cross-linking by *Trichoderma reesei* tyrosinase (TrTyr) and transglutaminase (TG). Changes in BLG molecular structure were determined at pH 6.8, 7.5 and 9.0 before and after high-temperature heat treatment. The conformational change was linked to efficiency of protein cross-linking. BLG was not susceptible to TrTyr without heat treatment. TG, however, induced inter-molecular cross-links at pH 7.5 and 9.0. After the heat treatments, BLG molecules adopted a molten-globule-like conformation. Both of the enzymes were able to form inter-molecular cross-links between heat-denatured BLG molecules. Electrophoretic mobility and broadness of the oligomer bands created by both enzymes on SDS-PAGE gels showed differences which were linked to the availability and number of target amino acid residues. Evidence for intra-molecular cross-linking was obtained. Once adsorbed to air/water interface, BLG formed a viscoelastic surface film which was characterized by surface shear rheology. Application of cross-linking enzymes under a dense layer of BLG molecules at the interface led to decreasing G' with time. Intra-molecular links were most probably favored against inter-molecular on packed BLG layer leading to constrained molecules. Results in general emphasize the importance of structural and colloidal aspects of protein molecules in controlling inter/intra-molecular bond formation by cross-linking enzymes.

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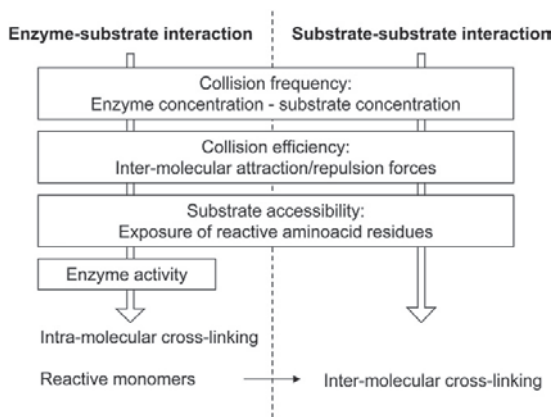
1. Introduction

Enzymatic cross-linking of food proteins has created increasing interest in recent years, the main argument being that the introduction of covalent links modifies the structure of the protein networks and that; due to the specificity of cross-linking enzymes, the properties of the structure formed can be controlled. In order to make efficient use of enzymes in food material modification, it is important to understand the availability of substrate protein for catalysis, i.e., the accessibility of reactive residues and also the probability of side-reactions. The various physical factors which are of importance in this respect are presented in Scheme 1. It has already been demonstrated that proteins without a confined tertiary structure are better substrates for cross-linking enzymes compared to globular proteins in their native form (Hellman,

Mattinen, Fu, Buchert, & Permi, 2011; Mattinen et al., 2006; Mattinen, Lantto, Selinheimo, Kruus, & Buchert, 2008).

The most abundant of whey proteins in bovine milk is β -lactoglobulin (BLG), which has a well-characterized globular structure. Under physiological conditions, bovine BLG exists as a homodimer consisting of ~ 18.3 kDa monomeric subunits. Each BLG monomer has 162 amino acids containing one free cysteine and two disulfide bridges 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 certain changes in tertiary structure (Brownlow et al., 1997; Considine, Patel, Anema, Singh, & Creamer, 2007; Qin et al., 1998). In its native state, BLG is resistant to enzymatic reactions. Its resistance to peptic enzymes, for example, is stated to be due to a highly stable, compact tertiary structure which hinders most of the specific peptides for cleavage (Reddy, Kella, & Kinsella, 1988). Similarly, enzymatic cross-linking of native BLG is limited possibly due to the hindrance of target amino acid side chains for cross-linking enzymes. In fact, complete

* Corresponding author. Tel.: +358 40 015 7616; fax: +358 20 722 7071.
E-mail address: dilek.ercili@vtt.fi (D. Ercili-Cura).



Scheme 1. Representation of conditions required for enzymatic cross-linking of proteins.

or partial denaturation of the molecule by physical or chemical means has been reported to increase the extent of protein cross-linking (Eissa, Puhl, Kadla, & Khan, 2006; Eissa, Satisha, & Khan, 2004; Færgemand, Murray, & Dickinson, 1997; Faegermand, Otte, & Qvist, 1998).

Exposure to different pH conditions, heat treatments, and adsorption to interfaces are all potential ways to induce conformational changes, which could then increase accessibility of BLG for enzymatic catalysis. In the region of Tanford transition, which is centered around pH 7.5, BLG molecule attains a molten-globule-like structure i.e., the protein retains its secondary structure and shows a compact tertiary structure but with increased mobility and looser packing thus increased reactivity (Tanford, Bunville, & Nozaki, 1959; Taulier & Chakilian, 2001; Yang, Dunker, Powers, Clark, & Swanson, 2001). At alkaline pH, BLG undergoes irreversible denaturation which induces monomer-aggregate equilibrium depending on protein concentration, ionic strength and temperature (Barteri, Gaudiano, Rotella, Benagiano, & Pala, 2000; Townend, Herskovits, Timasheff, & Gorbunoff, 1969). De Wit (2008) reviewed the thermal behavior of bovine BLG at temperatures up to 150 °C. Accordingly, between 60 and 70 °C (pH > 6.8) partial unfolding of BLG to molten-globule-like state and irreversible (to a limited extent) modification of monomers induced by exposed thiols were reported. At higher temperatures, increased unfolding followed by aggregation due to disulfide formation and hydrophobic interactions take place. Complete unfolding of BLG by breakdown of disulfide bonds can be achieved only after heating to temperatures as high as 125 °C (Watanabe & Klostermeyer, 1976). 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 patches align with the hydrophobic phase (Wilde, 2000). It is difficult to assess the exact conformation of adsorbed BLG molecules due to technical limitations in analysis methods, yet several studies have shown that the change in secondary structure is highly limited (Martin, Meinders, Bos, Cohen Stuart, & van Vliet, 2003; Meinders & de Jongh, 2002), and no decisive conclusions have yet been reported on tertiary structure.

Transglutaminase (TG, EC 2.3.2.13, γ -glutamyl-peptide, amine- γ -glutamyl transferase) is the most extensively studied protein cross-linking enzyme and has been thoroughly reviewed for its effects on dairy systems specifically (Jaros, Partschefeld, Henle, & Rohm, 2006). TG cross-links peptides and proteins through an acyl transfer mechanism between glutamine and lysine residues. In

the absence of amines, water serves as an acyl acceptor leading to conversion of glutamines to glutamic acid (deamidation) (Griffin, Casadio, & Bergamini, 2002; Kashiwagi et al., 2002). Oxidative enzymes as cross-linking enzymes are also gaining wider attention for the ability to oxidize protein substrates and subsequently modify the structural (Ercili-Cura et al., 2009, 2010; Lantto, Puolanne, Kalkkinen, Buchert, & Autio, 2005; Lantto, Puolanne, Kruus, Buchert, & Autio, 2007; Selinheimo, Autio, Kruus, & Buchert, 2007) and nutritional (Monogioudi et al., 2011; Stanic et al., 2010; Tantoush et al., 2011) properties of food products. Tyrosinases (EC 1.14.18.1) are mono-oxygenases which catalyze *ortho*-hydroxylation of monophenols to *o*-diphenols, which are further oxidized to *o*-quinones (Solomon, Sundaram, & Machonkin, 1996). Quinones are highly reactive and can further react non-enzymatically with each other or with thiol and/or amino groups resulting in formation of di-tyrosine, tyrosine-cysteine and tyrosine-lysine cross-links in protein substrates (Burzio, Burzio, Pardo, & Burzio, 2000; Ito, Kato, Shinpo, & Fujita, 1984; Matheis & Whitaker, 1984). Thus, modification of BLG molecule by tyrosinase depends mainly on the accessibility of tyrosine residues.

While the chemistry of enzymatically created cross-links is mainly known and a vast number of studies describe the effect of cross-linking on the macroscopic structure of a protein matrix (Buchert et al., 2010), relatively little is known about the physical constraints preceding enzymatic catalysis and the consequences of the links formed within the substrate molecules. The aim of this study was to relate pH- and temperature-induced conformational changes of BLG and constraints given by the environment, such as in solution vs. adsorbed layer, on its quality as a substrate for tyrosinase and transglutaminase catalyzed reactions.

2. Materials and methods

2.1. Preparation of BLG solutions

Bovine BLG (90% by PAGE, mixture of A and B variants) was purchased from Sigma (St. Louis, MO, USA) and used without further purification. Solutions of BLG were prepared at concentration of 1 or 2 mg mL⁻¹ at three different pH values; 6.8, 7.5 and 9.0. For pH 6.8 and 7.5, sodium phosphate buffer (20 mM) and for pH 9.0, borate buffer (20 mM) were used in all experiments. After dissolving, BLG solutions were left mixing for 1 h for equilibration. When needed, solutions were heat treated at 80 °C for 30 min (in water bath) or at 125 °C for 2 min (in a block heater). After the heat treatment, samples were directly put onto ice for 5 min and afterward tempered at room temperature. Before the analysis (CD or interfacial rheology), solutions were further diluted to desired concentrations and left at room temperature for 30–60 min for equilibration.

2.2. Circular dichroism (CD)

Changes in secondary and tertiary structure of BLG subjected to different pH conditions and heat treatments were assessed by CD. Spectra were recorded using a CD spectropolarimeter (JASCO J710, Jasco Ltd., Japan). Far-UV (180–260 nm) and near-UV (240–330 nm) CD spectra were recorded at room temperature with cells of 0.5 mm and 10 mm path lengths, respectively. Sample concentrations used for far- and near-UV were 0.2 mg mL⁻¹ and 2 mg mL⁻¹ respectively. Four accumulations were collected at 20 nm min⁻¹ scan speed, 1 nm bandwidth, 0.5 nm data pitch with 2 s response time during far-UV measurements. For near-UV, six accumulations were collected at 50 nm min⁻¹ scan speed, 1 nm bandwidth, 0.5 nm data pitch with 1 s response time. The instrument was calibrated using ammonium camphorsulfonic acid. Buffer baseline was subtracted

from each spectrum. The far-UV unsmoothed spectra are represented as molar CD [$\text{deg cm}^2 \text{dmol}^{-1}$] based on the mean residue molecular weight. The near-UV CD spectra are presented in measured ellipticities [mdeg] without smoothing.

2.3. Enzymatic modification

Trichoderma reesei tyrosinase (TrTyr) was produced at VTT as described by Selinheimo et al. (2006). Tyrosinase activity was measured spectrophotometrically by using 15 mM L-DOPA (Sigma, St. Luis, MO, USA) as substrate at pH 7.0 according to Robb (1984). The commercial Ca^{2+} -independent TG preparation (Activa[®] WM, Ajinomoto Inc., Japan) was supplied by Vesantti Oy (Helsinki, Finland) and further purified at VTT as described in Lantto et al. (2005). The activity of the TG preparation was measured by using 0.03 M N-carbobenzoxy-L-glutamyl-glycine as substrate at pH 6.0 according to Folk (1970). Accordingly, the specific activity of TrTyr was measured to be 425 nkat mg^{-1} and of TG was 500 nkat mg^{-1} . Enzymes were dosed based on the substrate protein concentration, i.e. nkat per g of protein in solution (nkat g^{-1}). Heat treated and untreated BLG solutions (1 mg mL^{-1}) were mixed with enzyme preparations at the dosage of 1000 nkat g^{-1} and incubated for 18 h under constant stirring either at room temperature or 40 °C respectively for TrTyr and TG. For TrTyr reactions, oxygen was continuously supplied by leaving the caps open under a closed container to limit evaporation.

2.4. SDS-PAGE analysis

The extent of BLG oligomerization caused by disulfide bridging was analyzed by non-reducing SDS-PAGE. Aliquots (65 μL) of sample solutions (1 mg mL^{-1}) were mixed with 25 μL of LDS sample buffer (glycerol, tris base, tris-HCl, lithium dodecyl-sulfate (LDS), EDTA, serva blue G250, phenol red) (4 \times) (Invitrogen Ltd., Paisley, UK) and 10 μL of distilled water for non-reducing PAGE. Enzyme-induced cross-linking of BLG was analyzed by reducing SDS-PAGE. Aliquots of enzyme-free control and enzyme-treated samples were mixed with 25 μL of LDS buffer and 10 μL of 50 mM DTT (Amersham Biosciences, Uppsala, Sweden) solution. Samples were heat treated at 70 °C for 10 min before loading to the gel. Non-reducing and reducing SDS-PAGE samples were loaded on 12% Bis-Tris gels (NuPAGE, Invitrogen Ltd., Paisley, UK) and run according to producer's protocol by using MOPS running buffer (20 \times) (Invitrogen Ltd., Paisley, UK). Gels were stained using coomassie (SimplyBlue SafeStain, Invitrogen Ltd., Paisley, UK). Mark12[™] unstained protein molecular weight standard (Invitrogen Ltd., Paisley, UK) was used in reducing SDS-PAGE gels. No molecular weight standard was shown in non-reducing page gel, however, the monomer and dimer bands were confirmed on another gel (data not shown).

2.5. Interfacial rheology

The surface shear rheological properties of BLG at the air–water interface were measured using a stress-controlled rheometer (AR-G2, TA Instruments, U.K.) equipped with a Pt–Ir du Noüy ring (13 mm diameter). The ring was flamed prior to each experiment. BLG stock solution (1 mg mL^{-1}), prepared at pH 6.8 was used with or without heat treatment (80 °C, 30 min). Stock solution was diluted 1:20 (0.05 mg mL^{-1}) in the same buffer (20 mM sodium phosphate at pH 6.8) before the rheological measurements. The instrument was mapped by performing rotational and oscillatory mappings prior to each measurement. Exactly 50 mL of prepared BLG solution was placed in a glass dish of 60 mm diameter and the du Noüy ring was placed onto the surface according to the manufacturer's instructions. Surface shear moduli were followed at 21 °C

at 0.1 Hz and constant strain of 0.5%, which was measured to be in the linear viscoelastic region (moduli were not dependent on the applied strain). The evolution of the moduli due to adsorption of BLG molecules were recorded during a 1 h time sweep. After that, the measurement was paused for 10 min and the prepared enzyme dilutions providing a dosage of 10 000 nkat g^{-1} (or 50 000 nkat g^{-1} for TG at non-heat-treated condition) for both TrTyr and TG were injected by using a 50 μL Hamilton glass injector to the subphase in small portions from several points. Enzyme dosage was increased to 10 000 nkat g^{-1} in interfacial measurements to compensate the effect of dilution on catalytic activity. Even if the local concentration of the substrate is high at the interface, no such gradient is expected in enzyme concentration. At the end of 10 min, another time sweep of 8 h was performed using the same parameters. Enzyme injection caused a reversible disturbance in BLG films as was observed in moduli evolution in time. To retard the evaporation, the measurement area was covered by the shield provided by the producer with several beakers of water inside and the open sides were sealed carefully with parafilm. The surface of the sample dish was also covered with a lid which does not disturb the measurement.

The interfacial shear rheological methods generally do not have good reproducibility (Krägel & Derkach, 2010). However, qualitative data can be achieved. As explained above, the protein adsorption and film formation was followed for 1 h for BLG alone before injection of the enzyme in each measurement. That way, a representative film was formed and the effect of any enzymatic action could be assessed qualitatively. Each measurement was replicated at least twice and the same trends were repeatedly observed upon enzyme treatment of BLG films.

2.6. Surface pressure measurements

The surface pressure of adsorbed BLG layers was measured with a KSV film balance (Minimicro series, KSV Instruments) using a platinum Wilhelmy plate at room temperature. The aim here was to create an adsorbed protein layer in the same conditions as in the rheological measurements. The same dish that was used for rheology measurements (60 mm diameter, glass) was first filled with 45 g of pure water at room temperature. The Wilhelmy plate (washed with ethanol, flamed and wetted prior to each experiment) was dipped into the water and the balance was zeroed at surface tension of around 72 mN m^{-1} . After this, the water was removed from the dish and the same amount of (45 g) BLG solution (0.05 mg mL^{-1}) prepared in the appropriate buffer was placed. The position of the Wilhelmy plate was not changed, thus the Wilhelmy plate was dipped to the same height in to the BLG solution as it was dipped in water. The surface pressure measurements started as soon as the BLG solution was in the dish and touched the Wilhelmy plate. Data was recorded in 60 sec intervals. After 1 h of adsorption, 45 μL of enzyme dilution, or only buffer for the control sample, was injected to the subphase in the same manner as for the rheological measurements. Surface pressure data acquisition was continued during enzyme injection, which again caused a reversible disruption in surface pressure. After the enzyme injection, measurements continued for 8 h. The experiments could be run for 9 h with negligible evaporation as the cabinet was sealed and several beakers filled with water were placed inside the cabinet.

3. Results

3.1. BLG conformation at different conditions

BLG conformation was analyzed at three different pH values: 6.8, 7.5 and 9.0, at which the solutions were either not heated or heated to 80 °C (30 min.) or 125 °C (2 min.). The lowest pH, 6.8 was chosen

as a reference as it is the physiological pH in which BLG is found naturally (the molecule is in the initial phases of Tanford transition at pH 6.8. We will still refer this state as 'native' throughout the text). The higher pH values; pH 7.5 and 9.0 were chosen as they represent the Tanford transition (Tanford et al., 1959) and the start of irreversible denaturation (Barteri et al., 2000; Townend et al., 1969), respectively. Far-UV spectra of the non-heated and heat-treated BLG solutions are shown in Fig. 1. At room temperature, far-UV spectra of BLG at both pH 6.8 and 7.5 showed a wide minimum at around 216 nm, typical of a predominantly β -sheet structure (Fig. 1A). At pH 9.0, a shift in negative peak was observed toward the region around 210 nm. Upon heating to 80 °C and subsequent cooling, the minimum peak was further shifted to the region below 205 nm at all pH values to the same extent (Fig. 1B). The same phenomena were observed upon heating at 125 °C although with a more pronounced effect at pH 9.0 (Fig. 1C). The CD spectrum of a random coil shows a single intense negative peak near 200 nm (Kelly, Jess, & Price, 2005). Observed effect of heat treatment on secondary structure is in accordance with other authors who reported loss of α -helices and β -sheets and increase in random coil structures upon heat treatment of BLG (Carrotta, Bauer, Waninge, & Rischel, 2001; Kim, Cornec, & Narsimhan, 2005). The effect of heat treatments for different pH values are depicted in Fig. 1D–F. At all pH values, the negative peak at ~205 nm was similar for both 80 °C and 125 °C heat treatments indicating no further loss of secondary structure upon 125 °C treatment as opposed to DSC data previously reported by De Wit (1981).

Near-UV CD spectra of BLG solution at the same pH conditions with/without heat treatment at 80 °C (30 min.) were also followed

(Fig. 2). Near-UV CD spectra of BLG at both pH 6.8 and 7.5 showed very intense negative peaks at 293 and 285 nm which are the typical features present in native folded conformation. No significant difference was observed between pH 6.8 and 7.5 in non-heat-treated samples which is in accordance with Taulier and Chakilian (2001). At pH 9.0, the intensity of negative bands at 285 and 293 nm were decreased compared to neutral pH in the non-heat-treated samples. This indicates a decrease in tertiary structure in the region of at least one of the two tryptophan residues. Upon heat treatment, those bands were significantly diminished at all pH values, with more significant change at pH 9.0 (Fig. 2). Lower negative ellipticity values were obtained at all wavelengths for heat-treated samples. The change was significant also between 260 and 280 nm. The loss of negative ellipticity in the near-UV CD spectra indicates a significant loss of tertiary structure. However, it should be kept in mind that there was 2–3 h in between the heat treatments and the CD analysis. Some extent of re-folding could have occurred during this period.

3.2. Oligomerization of BLG caused by inter-molecular disulfide bond formation

The degree of BLG oligomerization due to formation of inter-molecular disulfide bonds was analyzed by non-reducing SDS-PAGE (Fig. 3). Without any heat treatment, intense bands of BLG monomers and faint bands of BLG dimers are visible at all three pH values. This unusual dimer band (SDS-resistant dimer) was previously reported and was attributed to the BLG covalent dimers that

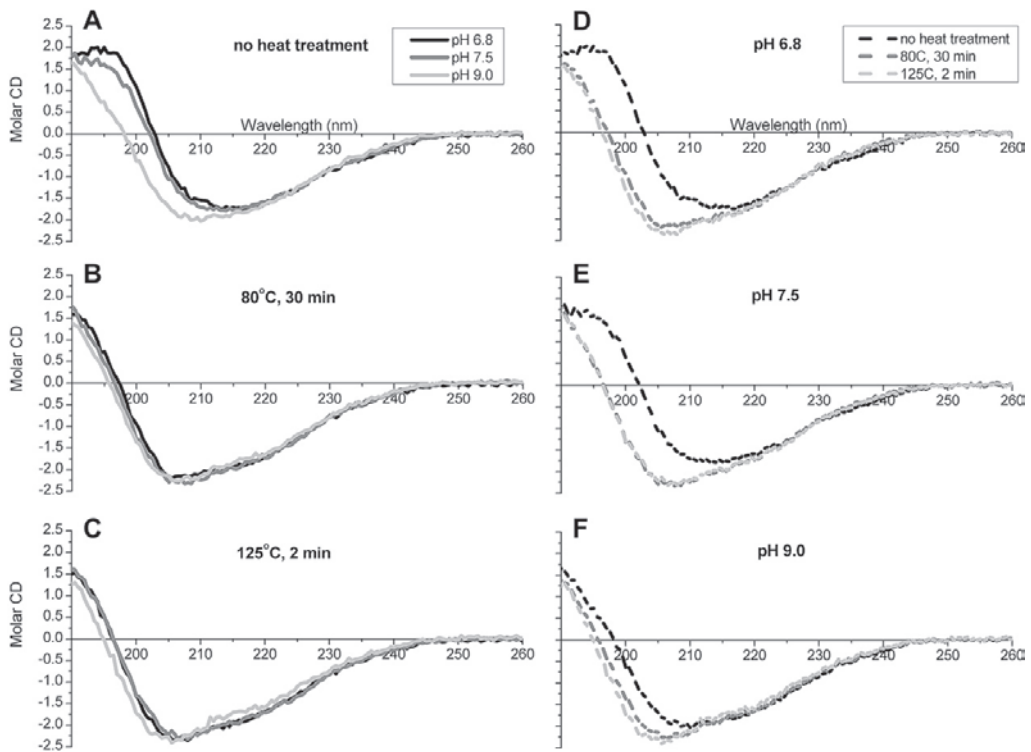


Fig. 1. Far-UV CD spectra of non-heated and heat-treated BLG molecules. Effect of pH at different heat treatments (A, B, C); effect of heating at different pH values (D, E, F). (D, E, and F are re-organization of the same data in A, B, C). The legends for the first and the second columns are shown in graphs A and D, respectively.

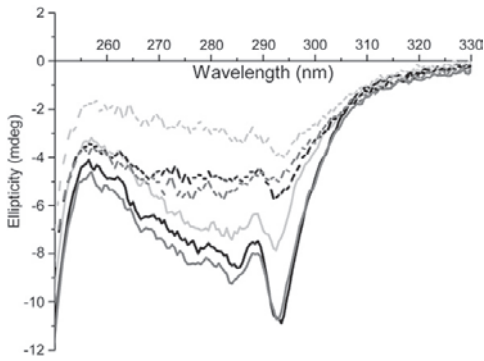


Fig. 2. Near-UV CD spectra of non-heated (dashed lines) and heat treated (solid lines) BLG molecules at pH 6.8 (black), pH 7.5 (gray), and pH 9.0 (light gray).

are naturally formed during processing and storage of such protein powders (de Jongh, Grönvelde, & de Groot, 2001; Muhammad, Croguennec, Julien, Michel, & Saïd, 2009). The figure shows that without heat treatment there were no disulfide-stabilized oligomers observed at pH 6.8 and 7.5. At pH 9, a slight smear could be seen around the SDS-resistant BLG dimer band. Exceptionally at pH 9.0 a faint band around 14 kDa is also visible (Fig. 3) which might be due to alkaline autolysis of BLG molecule (Christensen, 1949). After heat treatment, disulfide linked dimers, trimers and even bigger oligomers at both pH 6.8 and 7.5 for both 80 °C and 125 °C treatments were observed. However, at pH 9.0, only dimers were formed and to a relatively low extent for both heat treatments. There were no differences between the two heat treatments with respect to disulfide bond formation (Fig. 3). The majority of BLG remained monomeric in all cases.

3.3. Enzymatic cross-linking of BLG

For cross-linking reactions, BLG was incubated with TrTyr for 18 h at room temperature and with TG for 18 h at 40 °C. Room temperature was chosen for TrTyr as the enzyme is not very thermostable. It has a half-life of 15 min at 50 °C, and 18 h at 30 °C. TrTyr has its highest activity and stability at neutral and alkaline pH values with the optimum at pH 9 (Selinheim et al., 2006). TG is

reported to be stable at 40 °C which is also in the range of its optimum temperature. TG is stable at a pH range of 5.0–7.0 with an optimum at pH 6.0, but significantly loses activity outside of this pH range (Lu, Zhou, Tian, Li, & Chen, 2003).

Enzymatic cross-linking of BLG was analyzed by SDS-PAGE in reducing conditions. Enzymatic catalysis at different pH conditions (without heat treatment) is shown in Fig. 4. Without any heat treatment or enzyme addition, BLG monomers appear around 18 kDa together with a faint band at around 36 kDa, which belongs to the SDS-resistant dimer. Once more, a faint band at low molecular weight (14 kDa) is visible at pH 9.0 (Fig. 4). The TrTyr-treated BLG samples show no difference compared to control samples at pH 6.8 and 7.5. At pH 9.0 only faint smears below and above the SDS-resistant BLG dimer band could be observed after TrTyr treatment. When BLG was treated with TG at pH 6.8 no cross-linking was observed. However, at pH 7.5 oligomer bands which are scattered below and above 36 kDa are visible. Besides high molecular weight smears, a clear band below the BLG monomer at around 14 kDa was also formed. Similarly, at pH 9.0, a smeary band was visible below 36 kDa when treated with TG. Unlike pH 7.5, at pH 9.0 no smearing was observed above 36 kDa. Moreover, the band around 14 kDa was intensified and smeared upwards at pH 9.0 compared to the one at pH 7.5 in TG-treated samples (Fig. 4).

After the heat treatments at 80 °C or 125 °C, both TrTyr and TG treatments resulted in formation of covalent cross-links at all three pH values (Fig. 5A and B). The first three lanes of each gel in Fig. 5 represent the heat-treated controls (enzyme-free). No covalent oligomerization was observed without the presence of enzymes at any pH. Firstly, there were no differences in the patterns of oligomer formation due to either of the enzymes when comparing two heat treatment conditions. Therefore, Fig. 5A and B are analyzed as one. After heat treatment, TrTyr treatment at 1000 nkat g⁻¹ resulted in the formation of dimers, trimers and higher molecular weight oligomers at both pH 6.8 and 7.5 (Fig. 5A and B). At pH 9.0, degree of TrTyr cross-linking was decreased as some of the high molecular weight bands (>97 kDa) were missing or fainter compared to those at pH 6.8 and 7.5. Similarly, TG treatment at 1000 nkat g⁻¹ also resulted in protein cross-linking depicted by formation of intense bands at the high molecular weight range and a clear loss of intensity of the BLG monomers (Fig. 5A and B). However, the bands created by TG action were not exact but broad bands scattered significantly downwards on the gel, which was actually the case also in the TG-cross-linking products in the solution without heat treatment (Fig. 4). Dimers were observed as

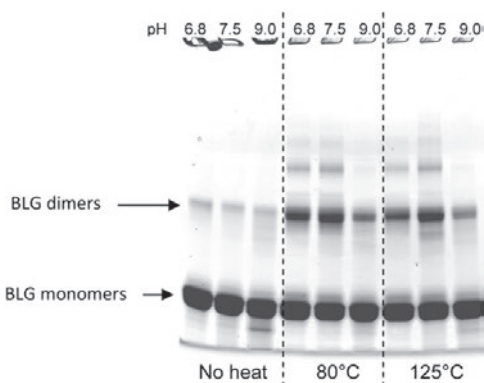


Fig. 3. Non-reducing SDS-PAGE of BLG at pH 6.8, 7.5 and 9.0; heat treated at 80 °C (30 min) and 125 °C (2 min) separately.

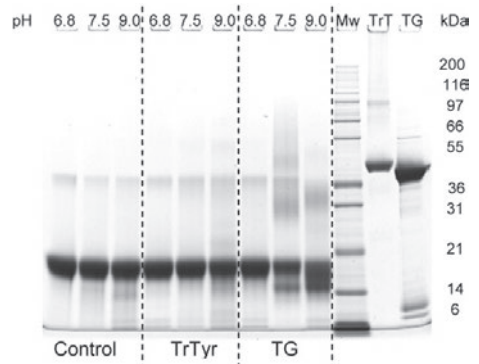


Fig. 4. Reducing SDS-PAGE of BLG at pH 6.8, 7.5 and 9.0 without any heat treatment. BLG was treated by TrTyr (1000 nkat g⁻¹) and TG (1000 nkat g⁻¹) for 18 h at RT and 40 °C, respectively. The last two lanes are TrTyr and TG loaded alone.

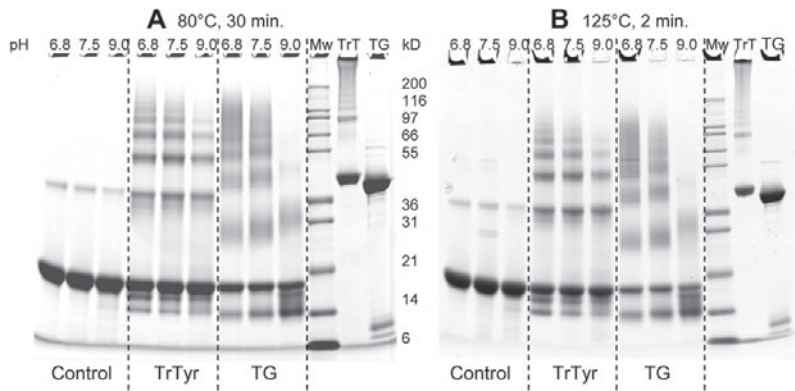


Fig. 5. Reducing SDS-PAGE of BLG prepared at pH 6.8, 7.5 and 9.0 that was heat treated at 80 °C, 30 min. (A) and heat treated at 125 °C, 2 min. (B). BLG was cross-linked by TrTyr (1000 nkat g⁻¹) and TG (1000 nkat g⁻¹) for 18 h at RT and 40 °C, respectively. The last two lanes, are TrTyr and TG loaded alone.

broad, smeared bands with an increased mobility compared to the original BLG dimer. They were identified at around 29 kDa instead of 36 kDa. The same phenomenon was observed for TG-induced trimers and higher oligomers. When the reaction was carried out at pH 9.0, TG-induced cross-linking was significantly limited compared to pH 6.8 and 7.5. Only a smeared dimer band again with an increased mobility (around 32 kDa) was observed. Besides the formation of higher molecular weight bands, in every lane that was treated by the enzymes, there were also low molecular weight bands which appear below the BLG monomer bands. More specifically, formation of two exact bands between 14 kDa and 18 kDa for the case of TrTyr and one exact band at 14 kDa for TG were observed at pH 6.8 and 7.5 after both heat treatments. At pH 9.0, those low molecular weight bands were rather broad and intensified in TG-treated samples (Fig. 5A and B).

The enzymes were also run separately on SDS-PAGE gels (Figs. 4 and 5, last two lanes). At the studied enzyme dosage, no bands due to the enzyme proteins were visible in sample lanes.

3.4. Interfacial properties

Interfacial shear rheological properties of both non-heat-treated and heat-treated BLG were measured. The aim was to analyze the enzymatic modification of BLG at the interface indirectly through the viscoelastic properties of the interfacial film. The results obtained by TG treatment only were shown as the results obtained by TrTyr were similar to TG.

BLG molecules adsorbed rather quickly to the air/water interface giving an elastic-dominant response (loss tangent (G''/G') < 1, not shown) immediately after the start of the measurements (Fig. 6). The initial surface shear elastic modulus (G') was around 0.020 N m⁻¹ at both non-heat-treated and heat-treated conditions (data not shown). The adsorption of BLG molecules were followed for 60 min until a representative film was formed. At the end of 60 min, enzyme or only buffer (for the control samples) were injected and the second time sweeps were started. The protein film was reversibly ruptured during the injections, as shown by a decrease in G' values in the initial phase of the second time sweeps which recovers in short time. In the case of the buffer injected controls, the film strength continued to grow until a final G' value that was ~1.2 times higher compared to the value that was attained at 60 min. In the case of non-heat-treated BLG, injection of TG at the dosage of 10 000 nkat g⁻¹ did not cause a considerable effect. The G' values leveled at a lower final G' compared to the

control, but not different compared to the 60 min value which was right before the enzyme injection. When the dosage of injected TG was increased to 50 000 nkat g⁻¹, a considerable (~23%) decrease in film strength was observed compared to the 60 min value (Fig. 6A).

Upon injection of 10 000 nkat g⁻¹ TG to the film formed by heat-treated BLG, G' continued to rise initially which was followed by

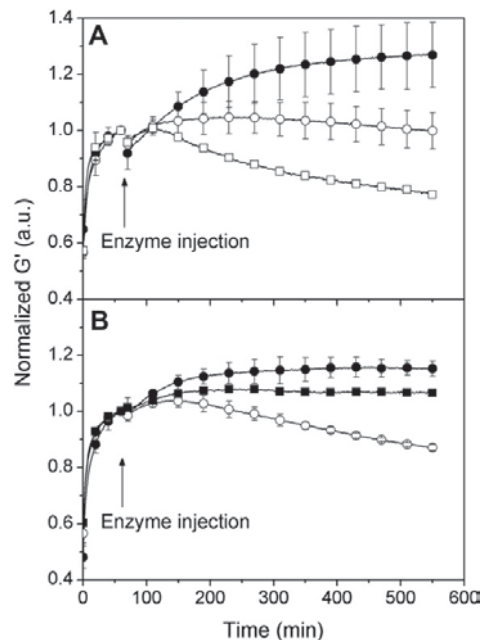


Fig. 6. Changes in interfacial shear elastic modulus (G') in time for non-heated (A); and heat treated (80 °C, 30 min) (B) BLG solutions at pH 6.8. BLG adsorption was followed for 1 h, after which 10 000 nkat g⁻¹ transglutaminase (TG) was injected to the subphase (○). Only buffer was injected to the control samples (●). In addition, a curve with 50 000 nkat g⁻¹ TG injection (■) was shown in (B). Vertical bars represent standard deviation. Values were normalized to the G' values at 60 min (right before enzyme or buffer injection) for each sample.

a noticeable decrease after 150–200 min of measurement time. The G' of TG-treated sample was decreased ($\sim 13\%$) at 550 min compared to the value at 60 min (Fig. 6B). When the BLG molecules were more enzyme-susceptible (heat-treated case), the effect reflected on the interfacial shear elastic modulus was higher compared to the more native state (non-heat-treated case) for the same enzyme dosage. Overall, there was a negative effect of the enzyme-treatments on development of G' which was more evident when BLG was heat treated. The probable effect of adsorption of enzyme protein itself was also tested by conducting the experiments in the same manner but using inactivated enzyme preparations. TG was inactivated by incubation at 50°C for 18 h. No considerable effect due to adsorption of the TG proteins or any contaminating surface active component was detected (Fig. 6B).

To obtain more insight on the adsorbed films before and after the enzyme injections, the saturation of the air–water interface was followed by surface pressure measurements conducted in parallel with the rheological measurements. Results representative of the samples in Fig. 6B are depicted in Fig. 7. The initial surface pressure values were already very high ($\sim 20\text{ mN m}^{-1}$) and did not change much in the course of 9 h. The disruption of the film caused by enzyme or buffer injections was quickly recovered for all samples and no significant effect of the enzyme protein added to the solution was reflected to surface pressure (Fig. 7).

4. Discussion

As BLG is rather resistant to enzymatic modification in the native state, an attempt was made to relate conformational changes induced by pH and heating to its susceptibility toward transglutaminase and tyrosinase. Attention is given to the physical constraints in formation of intra- and/or inter-molecular links, either caused by protein conformation or by its environment, bulk or interfacial systems.

The far-UV results showed β -sheet-dominant structures at all pH values studied (without heat treatment) with a slight change only at pH 9.0. In near-UV spectra, the intensity of minimum peaks was decreased at pH 9.0 compared to neutral pH indicating that the specific and rigid packing of aromatic residues, namely tryptophan and tyrosine, was partly lost at pH 9.0. Interestingly, TG-induced cross-linking was possible at pH 7.5 without any heat treatment (Fig. 4), and was thus not linked with the minor changes in secondary or tertiary structures observed only at pH 9.0. The only

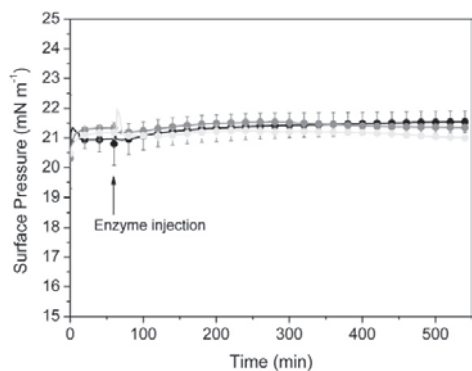


Fig. 7. Changes in surface pressure for heat-treated BLG solution at pH 6.8. Adsorption was followed for 1 h, until the point of 'enzyme injection' at which $10\,000\text{ nkat g}^{-1}$ of TrTy (gray) or TG (light gray) was injected to the subphase. Only buffer was injected to the control sample (black). Vertical bars represent standard deviation.

conformational change in BLG at around pH 7.5 that could explain this behavior is a local displacement of the EF loop opening the calyx interior (Qin et al., 1998) and the shift in monomer–dimer equilibrium toward monomeric form. Neither of these can be detected by far- or near-UV CD (Taulier & Chakilian, 2001). At pH 7.5, reactivity of 4 glutamines and 3 lysines in BLG has been reported previously (Nieuwenhuizen, Dekker, Gröneveld, de Koster, & de Jong, 2004), but as none of these identified residues can be directly associated with the dimer interface or the EF loop, it is not evident why they become reactive. 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).

Tyrosinase, which is still active at pH 9.0, was unable to catalyze cross-linking at any of the pH values in unheated BLG. This was most likely because there are 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 and two tyrosines are buried (Brownlow et al., 1997; Townend et al., 1969). The loosening of the three-dimensional structure and increased backbone flexibility of BLG molecule at pH 9.0 has previously been associated with increased reactivity toward tyrosinase (Partanen et al., 2011). Compared to the present study, a higher substrate concentration was used by Partanen et al., which could well indicate the co-existence of a minor fraction of reactive conformation and a major non-accessible fraction.

Upon heat treatments, a similar decrease in ordered secondary structure with increase in random coil in far-UV spectra and a significant loss of tertiary structure around the tyrosine and tryptophan residues in the near-UV spectra was found, which enabled cross-linking of BLG by both enzymes. Cross-linking of BLG upon loss of native structure is in agreement with previous reports showing the need to denature either by means of heat (Eissa et al., 2006; Sharma, Lorenzen, & Qvist, 2001), high pressure (Lauber, Krause, Klostermeyer, & Henle, 2003) or chemical agents (Eissa et al., 2006; Færgemand et al., 1997, 1998) for significant susceptibility of BLG to cross-linking. After heat treatment, there was no difference between pH values 6.8 and 7.5 in the patterns of formed oligomers by either of the enzymes, but pH 9.0 was clearly least efficient in catalyzing formation of inter-molecular cross-links. This could be mainly due to the effect of pH in activity and stability of the enzymes: pH 9.0 was reported to be the optimum pH for TrTy (Selinheimo et al., 2006), but there is not much data about its stability at that pH. And for TG, pH 9.0 is rather high as was also discussed for the non-heat-treated case.

An interesting outcome of the SDS-PAGE patterns of heat-treated BLG was the difference observed between the shape and mobility of bands after incubation with TrTy and TG (Fig. 5). The broadening and increased mobility 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 higher number of reactive residues for TG as compared with TrTy. It should also be noted that in the absence of available lysines in close proximity of glutamines, TG action induces deamidation reaction. As a consequence, glutamines are transformed into glutamic acid residues, which in turn lower the pI of BLG (Nieuwenhuizen et al., 2004) causing more heterogeneity. Another explanation for increased band mobility could be extensive levels of intra-molecular cross-linking which might affect overall shape of the SDS-denatured molecule. Hellman et al. (2011) have recently shown that a model globular protein was not inter-molecularly cross-linked by tyrosinase unless partially denatured (or unless having water accessible tyrosine close to a nucleophilic amino acid in the folded form). However, intra-molecular cross-linking was still prevalent which induced formation of non-native monomers

which showed delayed elution in size exclusion chromatography. Accordingly, we suggest that the low molecular weight bands observed below the BLG monomers (~14 kDa) could as well belong to intra-linked, or alternatively, in the case of TG, deamidated monomers. The fact that the low molecular weight bands were intensified at pH 9.0 could be due to physical constraints of BLG molecule at that pH. For example, lower amounts of disulfide linked dimers were observed at pH 9.0 compared to other pH values which might favor internal cross-linking of monomeric subunits. Finally, those low molecular weight bands are not caused by protease contaminations in enzyme preparations as both TrTyr and TG were tested negative for proteolytic activity at neutral pH (data not shown).

Interfacial shear rheology is a good approach to analyze the intra- and/or inter-molecular interactions at interfaces (Krägel & Derkatch, 2010). Our driving idea for using enzymatic cross-linking at an adsorbed layer of BLG was the reported behavior of BLG to partially unfold once adsorbed to air/water interface (see reviews by Bos & van Vliet, 2001; Murray, 2002; Wilde, 2000). Many reports have been published on unfolding kinetics of globular proteins at interfaces with a common conclusion that partial unfolding upon self-re-arrangement of adsorbed proteins may happen if the protein has a certain minimum area to expand. Surface pressure measurements revealed that at the BLG concentration studied, adsorption of protein molecules to the surface was rather fast. In fact, the surface pressure reached ~20 mN m⁻¹ within seconds. Even though the interface was saturated with BLG molecules instantly, G' continued to increase with time. That implies on-going structural organization with increasing lateral interactions between adsorbed molecules. Enzymes were injected underneath the packed protein layer. After addition of TG to the subphase, firstly the film recovered from the injection damage similar to control, but in time, film strength was lowered as was observed by decreasing G' . We would expect that creation of inter-molecular covalent bonds by the enzymes would increase the film strength. In fact, Færgemand and Murray (1998) have reported increased surface dilatational elastic modulus upon TG treatment of adsorbed BLG molecules. On the other hand, Romoscanu and Mezzenga (2005) showed that glutaraldehyde-induced cross-linking increased the elastic modulus of non-densified BLG interface while the effect was reversed when glutaraldehyde was applied on densified, folded interface. Accordingly, we may claim that, once adsorbed fast at such high surface concentration, BLG molecules attained a constrained structure which limited formation of enzyme-induced inter-molecular covalent links. However, intra-molecular links could well be formed within the adsorbed molecules. Hellman et al. (2011) have recently shown that intra-molecularly cross-linked proteins are locked in their globular fold. Such bonds created by both TrTyr and TG would then further impede the re-arrangement of protein adsorbed to the interface during aging which would lead to diminishing of physical protein–protein interactions at the interface. Eissa et al. (2006) reported that formation of compact molecules by TG action limits exposure of hydrophobic regions thus attenuates hydrophobic interactions in whey proteins which might similarly limit further development of hydrophobic interactions between the adsorbed proteins and lead to decreasing film strength. Another argument for TG action could be the deamidation which would increase the negative charge of the protein molecules leading to weakening of inter-molecular interactions as a result of increased electrostatic repulsion.

There are several techniques used for detection of the conformation of adsorbed proteins but these are still being developed. So far, the evidence for change of tertiary structure was most frequently obtained through indirect methods such as following

the change in surface pressure, surface viscosity or viscoelasticity in time which would infer evidence for inter-molecular interactions and a change of fold. Interpretation of the indirect evidence attained depends, however, on two assumptions as was made clear by Wierenga and Gruppen (2010) recently: 1. To consider proteins as flexible polymer chains that adopt different structures depending on the surface load or pressure, 2. To consider proteins as colloidal particles similar to hard spheres which preserve structural integrity upon adsorption. Accordingly, it is challenging to fully understand what gives the elastic response in such systems and how the film strength can be tailored for e.g. improved foam stability.

5. Conclusions

Subjecting BLG to slightly alkaline (pH 7.5) and alkaline (pH 9.0) pH conditions led to enzymatic cross-linking by transglutaminase. This limited cross-linking could be due to a minor population shifting toward a molten-globule state, as little change in secondary or tertiary structures was found at pH 7.5. Upon heat treatment and cooling, formation of covalently linked BLG oligomers was associated with molten-globule-like conformation of the substrate which shows as a small change in secondary structure and a significantly disturbed tertiary structure. As with monomers, increased mobility of transglutaminase-induced oligomers on gel electrophoresis is suggested to be due to extensive internal cross-linking. The smearing of the bands is explained by the higher number of reactive residues for TG as compared with TrTyr, and therefore an increased number of possible combinations of inter-molecular cross-links leading to formation of heterogeneous oligomers. When heat-treated BLG molecules adsorbed to air/water interface were enzyme-treated, the shear elastic modulus decreased with time. We suggest that due to rapid saturation of the surface, the mobility of the BLG becomes a limiting factor for inter-molecular cross-linking. However, intra-molecular links which make the adsorbed molecules even more rigid and less free to reorganize still occur. As a whole the results show the role of colloidal interactions and physical constraints in controlling the formation of enzyme-induced inter-molecular protein cross-linking.

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PUBLICATION II

**Laccase-aided protein
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Laccase-aided protein modification: Effects on the structural properties of acidified sodium caseinate gels

D. Ercili Cura*, R. Lantto, M. Lille, M. Andberg, K. Kruus, J. Buchert

VTT Technical Research Centre of Finland, VTT, P.O. Box 1000, FI-02044 Espoo, Finland

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ABSTRACT

Trametes hirsuta laccase (EC 1.10.32) alone was not able to cross-link proteins effectively unless high dosages were used. Incorporation of ferulic acid enhanced the formation of intermolecular cross-links. Cross-linking was more effective when the reaction was carried out at 45 °C rather than at room temperature. Size exclusion chromatography showed that ferulic acid monomers disappeared from the solution in the presence of laccase. Force at rupture point of the caseinate gels treated with laccase without ferulic acid was not significantly different from the control, while stronger gels were achieved when ferulic acid was present. Rheological measurements showed that laccase treatment together with ferulic acid resulted in higher G' values than the control. Gels were analyzed with confocal laser scanning microscope. A finer network was observed when the enzyme was used together with ferulic acid. Slight proteolytic activity resulted in a negative effect on the gel firmness and microstructure when laccase was used without ferulic acid.

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1. Introduction

Enzyme-aided structure engineering of dairy products has been of much interest since the introduction of the protein cross-linking enzyme transglutaminase (glutaminylpeptide:amine γ -glutamyltransferase, EC 2.3.2.13, TG), a transferase capable of forming inter- or intramolecular isopeptide bonds in protein systems (Jaros, Partschfeld, Henle, & Rohm, 2006; Jaros, Pätzold, Schwarzenbotz, & Rohm, 2006). TG-aided cross-linking of caseins has been reported to lead to milk protein gels with firmer texture (Anema, Lauber, Lee, Henle, & Klostermeyer, 2005; Lauber, Henle, & Klostermeyer, 2000) and less syneresis (Bönisch, Huss, Weitzl, & Kulozik, 2007; Myllärinen, Buchert, & Autio, 2007; Schorsch, Carrie, Clark, & Norton, 2000). In addition to TG, other types of cross-linking enzymes are suitable for protein cross-linking as reviewed previously by Buchert et al. (2007).

Laccases (benzenediol oxygen oxidoreductase, EC 1.10.3.2) are copper-containing oxidases that 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. In higher plants laccases are involved in cell wall formation and lignin biosynthesis (Gianfreda, Xu, & Bollag, 1999; Thurston, 1994), whereas fungal laccases are involved in

lignin degradation (Mayer & Staples, 2002; Thurston, 1994; Xu, 1996). In insects laccases are reported to be involved in cuticle sclerotization by catalyzing oxidative coupling of catechols with proteins (Kramer et al., 2001).

Laccases can act on a broad range of substrates including diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, benzenethiols and even some inorganic compounds such as iodine (Gianfreda et al., 1999; Mayer & Staples, 2002; Thurston, 1994; Xu, 1996). Laccases oxidize their substrates with a one-electron removal mechanism, producing free radicals that can further undergo non-enzymatic reactions leading to polymerization, hydration, and fragmentation (Claus, 2003; Thurston, 1994). The capability of laccases to oxidize a wide variety of phenolic and other substrates has raised interest in their applicability for protein cross-linking or modification (Chung, Kurisawa, Uyama, & Kobayashi, 2003; Færgemand, Otte, & Qvist, 1998; Heldt-Hansen, 1998; Lantto, Schönberg, Heine, & Buchert, 2004). In many studies, the reactivity of laccases has been enhanced by the presence of a low molecular weight phenolic component. α -Lactalbumin and denaturated β -lactoglobulin have been polymerized by *Polyporus pinsitus* laccase in the presence of chlorogenic acid (Færgemand et al., 1998). Yamaguchi (2000) reported polymerization of caseins and bovine serum albumin (BSA) by *Pycnoporus* laccase. Mattinen et al. (2006) recently reported *Trametes hirsuta* laccase-catalyzed oligomerisation of coactosin without an auxiliary substance although it was concluded that, in general, proteins are poor substrates for laccase.

* Corresponding author. Tel.: +358 40 015 7616; fax: +358 20 722 7071.
E-mail address: dilek.ercili@vtt.fi (D. Ercili Cura).

Laccase-catalyzed oligomerization of peptides or proteins is reported to proceed through oxidation of tyrosine, cysteine or tryptophan residues (Figueroa-Espinoza, Morel, & Rouau, 1998; Labat, Morel, & Rouau, 2000; Mattinen et al., 2006; Tsuchiya, Petersen, & Christensen, 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). Mattinen et al. (2005) also showed that isodityrosine bonds are formed when hydroxyl and tyrosyl radicals located in different molecules reacted 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 high redox potential of proteins (Xu, 1996). Thus the presence of a mediator or auxiliary substance is needed. The auxiliary substances or mediators are most often small molecules that are readily oxidized by laccase, producing radicals which can then react with the target substrate.

Ferulic acid (FA) is a small phenolic compound that is easily oxidized by laccase (Figueroa-Espinoza & Rouau, 1998; Figueroa-Espinoza et al., 1998; Labat et al., 2000; Selinheimo, Autio, Kruus, & Buchert, 2007; Selinheimo, Lampila, Mattinen, & Buchert, 2008). The proposed mechanism for FA oxidation by laccase is the dehydrogenation of the hydroxyl group at C-4 of FA into reactive phenoxy radicals that can further dimerize or react with another radical to form C-C or C-O linkages (Ralph, Quideau, Grabber, & Hatfield, 1994; Thomson, 1964). The phenoxy radical has been proposed to be able to oxidize the phenolic group of tyrosine (Neukom & Markwalder, 1978) or the thiol group of cysteine (Figueroa-Espinoza et al., 1998; Labat et al., 2000). As a result protein-protein interactions are reported to be obtained.

A caseinate gel is formed during acidification due to the loss of electrostatic repulsion and aggregation of the caseins. Acidification of non-enzyme-treated caseins is known to induce formation of mainly physical bonds (Braga, Menossi, & Cunha, 2006; Dickinson, 2006). The aim of this study was to elucidate the potential of a white-rot fungus *T. hirsuta* laccase in the modification of casein proteins and investigate the effects of newly formed covalent bonds on acidified sodium caseinate gel structures. Consequences of casein cross-linking with or without FA and the role of proteolytic side activity in the laccase preparation were investigated by following rheological and microstructure properties of acidified sodium caseinate gels.

2. Materials and methods

2.1. Chemicals

Sodium caseinate (protein content 90%) was supplied by Valio Ltd (Helsinki, Finland). D-glucono- δ -lactone (GDL) (G4750) was purchased from Sigma Chemicals (St. Louis, MO, USA). Ferulic acid was purchased from Fluka (Buchs, Switzerland). CHAPS (3-[3-cholamidopropyl] dimethylammonium)-1-propanesulfonate) and 1,4-dithiothreitol (DTT) were obtained from Sigma Chemicals, while Rhodamin B was from Merck (Darmstadt, Germany). All other chemicals were of the analytical grade.

2.2. Enzyme and enzyme activity measurements

Laccase was produced by *T. hirsuta* and partially purified by anion exchange chromatography (laccase A). The partially purified laccase was further purified by hydrophobic interaction chromatography (laccase B) (Rittstieg et al., 2002). Laccase activity was measured

using two different assays: a spectrophotometric method based on 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) as substrate (Niku-Paavola, Karhunen, Salola, & Raunio, 1988) and the oxygen consumption method by using ferulic acid as substrate (Selinheimo et al., 2007). pH of the former assay was 4.5. Oxygen consumption was followed by using a single channel oxygen meter (Fibox 3, Precision sensing GmbH, Germany) with FA as substrate at a concentration of 10 mM in 0.1 M sodium phosphate buffer (pH 7.0). Activity was determined by following the consumption of oxygen in a closed and fully filled vial (1860 μ L) at room temperature. Reaction was initiated by addition of 20 μ L of enzyme to the substrate solution. The activity on FA was calculated from the amount of O₂ consumed (4 molecules of FA oxidized when 1 molecule of dioxygen consumed (Claus, 2004; Solomon, Sundaram, & Machonkin, 1996). In all experiments, laccase was dosed based on the activity measured by oxygen consumption method.

Possible protease activity in the laccase preparations was measured using two different assays: QuantiCleave™ fluorescent protease assay kit (Pierce, Rockford, IL, USA) and endo-protease assay using Protozyme AK tablets (Megazyme International Ireland Ltd., Bray, Ireland). Prior to the protease activity assays the laccase activity was inactivated by adding 100 μ M sodium azide to the preparation. In the former assay, protease activity is detected by an increase of fluorescence during hydrolysis of the fluorescein-labelled casein (FTC-casein). Trypsin was used as a standard. Protease activity measurement with Protozyme AK tablets was performed at pH 7.0 with modified reaction times. The substrate used in the assay was azurine-cross-linked casein (AZCL-casein). During hydrolysis water soluble dyed fragments absorbing at 590 nm are liberated from the substrate due to the protease activity.

Enzyme activities are expressed as specific activity, i.e., nanokatal per milligram (nkat mg⁻¹) of the enzyme protein in preparation. A single nkat is defined as the amount of enzyme required to convert 1 nmol s⁻¹ of the substrate used in the assay conditions.

2.3. Gel preparation

Sodium caseinate solution (5% w/w) was prepared by mixing sodium caseinate powder in distilled water with a magnetic stirrer overnight at +4 °C and finally tempering to room temperature. Samples with laccase with/without FA were prepared by mixing the sodium caseinate solutions at either room temperature (RT) or 45 °C for 2 h. The reaction mixtures were continuously supplied by oxygen by bubbling (gas mixture: 80% O₂, 20% N₂). Amount of enzyme preparation to be added to the substrate solution was determined based on the enzyme activity (measured by oxygen consumption) and the protein concentration of the substrate solution. Accordingly, two dosages, 2.5 and 25 nkat of laccase per gram of sodium caseinate were used. Concentration of FA was kept constant (2.5 mM). After the laccase reactions, samples which were incubated at 45 °C were tempered quickly to room temperature and the solutions were acidified by addition of 1.13% (w/w) GDL and stirred until GDL was totally dissolved. All the samples were acidified at 25 °C and incubated for 22 h to a final pH of 4.2. Laccase was not inactivated either before or after gel formation.

2.4. SDS-PAGE analysis

Acidified caseinate gels were freeze-dried after 22 h of incubation and dissolved in 0.1 M sodium phosphate buffer (pH 7.0) to a concentration of 3 mg mL⁻¹ prior to SDS-PAGE analysis. SDS-PAGE was performed according to Laemmli (1970). A Bio-Rad electrophoresis unit (Bio-Rad Laboratories, Richmond, CA, USA) and ready-made 12% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA, USA) were used. Protein bands were visualized by staining with

Coomassie (Serva Blue R, Serva Electrophoresis, Heidelberg, Germany). No molecular weight standards were run as molecular weights of the modified proteins were not intended to be estimated due to the anomalous behaviour of caseins on SDS-PAGE (Creamer & Richardson, 1984).

2.5. Size exclusion chromatography (SEC)

Freeze-dried caseinate gels were used for SEC analysis. SEC was performed according to Bönisch, Lauber, and Kulozik (2004). An ÄKTA purifier liquid chromatography system together with a Superdex 200 HR 10/30 gel filtration column (both from Amersham Pharmacia Biotech, Uppsala, Sweden) was used. Elution was done at room temperature at a flow rate of 0.5 mL min⁻¹ with 0.1 M sodium phosphate buffer pH 6.8, containing 6 M urea, 0.1 M sodium chloride, and 0.1% CHAPS. Freeze-dried caseinate samples were dissolved at a concentration of 0.1% in the elution buffer, containing 1% DTT as a reducing agent and kept overnight mixing at 4 °C. Sample injection volume to the column was 100 µL. Prior to injection samples were filtered through a 0.45 µm membrane (Syringe Driven Filter unit, 25 or 4 mm, Millex-HA, Millipore, Billerica, MA, USA). Protein detection was performed with a UV detector at 280 nm.

2.6. Large deformation measurements

The firmness of the caseinate gels were analyzed with a TA-HDi Texture Analyser (Stable Microsystems, Ltd., Godalming, England) equipped with a 5 kg load cell. Gels were tested directly after incubation at 25 °C for 22 h by penetration with a hemispherical plastic probe (∅ 1.27 cm) at a constant speed of 0.5 mm s⁻¹ to a distance of 70% of the gel height. The diameter of the gel was 3.7 cm and gel height was 1.5 cm. Force at the rupture point was recorded as a measure of the gel firmness. Measurements were performed on six individual gels for each sample.

2.7. Small deformation measurements

The formation of sodium caseinate gels was followed using a stress-controlled rheometer (StressTech, Reologica Instruments AB, Lund Sweden) in dynamic oscillation mode. Samples were placed into the concentric cylinder measuring system exactly after 20 min of GDL addition. All measurements were carried out at 25 °C at a frequency of 1.0 Hz. Strain was kept constant at 0.01 which was tested to be in the linear viscoelastic region. Gel formation was followed for 5 h after GDL addition after which a frequency sweep from 0.01 to 100 Hz was performed. In situ gelation was repeated two times for each sample.

2.8. Microscopy

The microstructure of the samples was examined with a Confocal Laser Scanning Microscope (CLSM) (BIO-RAD, Hemel Hempstead Herts, England with Argon Laser 488 nm; emission filter, E570LP). Samples were prepared by mixing 10 mL of the acidified sodium caseinate solution with 100 µL of the fluorescent dye Rhodamin B solution (0.1% w/w). Then 300 µL of the stained solution was pipetted onto a microscopy slide with Press-to-Seal™ (Molecular Probes, Eugene, OR, USA) silicone isolator with adhesive (20 mm diameter, 1.0 mm deep) surface. Slides with sample solutions were covered with cover glass and sealed with nail polish. Samples were incubated for gel formation at the same conditions (22 h at 25 °C) and examined directly after incubation. The micrographs were acquired at 512 × 512 pixel resolution and the final image was an overlay of 9 scans (in 2 µm depth) at the same focal plane. The structures obtained were reproducible.

2.9. Statistical analysis

Results were expressed as mean values of 6 individual measurements for large deformation (force at rupture) results. One-way ANOVA followed by an appropriate comparison of mean values was used (SPSS 14.0, SPSS Inc., Chicago, IL, USA). Values were considered to be significantly different at $p < 0.05$. All other measurements were replicated at least twice.

3. Results and discussion

3.1. Characterization of the two laccase preparations

The activity profiles of the two laccase preparations were first characterized (Table 1). Laccase activity was measured with two different methods, i.e., ABTS activity and FA oxidizing activity. The specific ABTS activities of the laccase preparations A and B were 1400 nkat mg⁻¹ and 2500 nkat mg⁻¹ and corresponding FA oxidation activities were 82 nkat mg⁻¹ and 87 nkat mg⁻¹, respectively. Fungal laccases are reported to have a pH optimum around 4–6 with phenolic substrates. The activity decreases with increasing pH due to inhibitory effect of hydroxide ions (Xu, 1997). On the other hand, the increasing pH decreases the redox potential of the phenolic substrate making it more susceptible to oxidation by laccase (Xu, 1997). In contrast to their activity, laccases as such are generally more stable at alkaline pH than at acidic pH (Xu, 1999). Laccase activity is mostly measured and reported on chromogenic substrates such as ABTS, which is very suitable as a substrate because its oxidation product is soluble in water, stable and colored (Xu, 1999). In this study, laccase activity was also tested on ferulic acid at pH 7.0 as it would give a more realistic view of the activity of the enzyme in the experimental conditions.

Possible protease activity in the preparations was also measured. According to the QuantiCleave™ fluorescent protease assay, a fluorescence value equivalent to 0.002 mg trypsin was detected per mg protein in laccase A, and according to the endo-protease assay (Protozyme AK Tablets) a protease activity of 0.05 nkat mg⁻¹ was detected. Laccase B had no measurable protease activity with either of the methods used. Protease activity assays were performed in the presence of sodium azide to inactivate the laccase and make sure that proteolytic activity was due to protease contamination rather than possible laccase reactions. The protease impurity of laccase A was very low, but it might have an impact on the caseinate gels in prolonged incubation times.

3.2. Modification of sodium caseinate by laccase and FA

Sodium caseinate solution was pre-treated at either room temperature (RT) or 45 °C for 2 h separately by both laccase A and B at the dosages of 2.5 and 25 nkat g⁻¹, with or without FA. After the enzyme treatments, caseinate solutions were acidified by GDL and incubated at 25 °C for 22 h. Resulting caseinate gels were freeze-dried and prepared for SDS-PAGE to analyze the extent of protein modification (Fig. 1). SDS-PAGE gels of samples that were pre-treated by laccase A at RT and 45 °C are depicted in Fig. 1a and b respectively, while SDS-PAGE gels of laccase B treated samples are shown in Fig. 1c and d.

In the absence of FA, laccase A was not cross-linking caseins at low dosage (2.5 nkat g⁻¹) irrespective of the pre-treatment temperatures (Fig. 1a and b, lane 3) but slight strengthening of high molecular weight protein bands was observed for sodium caseinate pre-treated with high enzyme dosage (25 nkat g⁻¹) (Fig. 1a and b, lane 4). FA enhanced the formation of larger reaction products at both pre-treatment temperatures (Fig. 1a and b, lanes 5 and 6). At 45 °C, which is close to optimum temperature of *T. hirsuta* laccase

Table 1
Enzyme activity profiles of the *T. hirsuta* laccase preparations.

Enzyme	Protein content (mg mL ⁻¹)	Laccase activity at pH 4.5 ^a (nkat mg ⁻¹)	Laccase activity at pH 7.0 ^b (nkat mg ⁻¹)	Protease content ^c (mg mg ⁻¹)	Protease activity ^d at pH 7.0 (nkat mg ⁻¹)
Laccase A	3.90	1436	82	0.002	0.050
Laccase B	4.86	2509	87	nd ^e	nd

^a Spectrophotometric method by using ABTS as substrate (Niku-Paavola et al., 1988).

^b Oxygen consumption method by using FA as substrate (Selinheimo et al., 2007).

^c Trypsin equivalent (QuantiCleave™ fluorescent protease assay kit (Pierce, Rockford, IL).

^d Protease activity according to trypsin (assay of endo-protease using Protozyme AK tablets (Megazyme International Ireland Ltd., Bray, Ireland).

^e Not detected.

(Rebrikov et al., 2006; Shleev et al., 2004), the reaction was much faster and higher extent of protein polymerization could be observed compared with when the reaction was carried out at room temperature. Treatment of sodium caseinate with laccase A resulted in formation of polymerization products, but also formation of low molecular weight protein bands was clearly observed with both enzyme dosages. Some of the fragmentation bands were observed to disappear in the samples pre-treated at 45 °C indicating that at that temperature, reaction is faster and leads to cross-linking of protein fragments in addition to cross-linking of monomeric caseins.

In the SDS-PAGE of laccase B treated sodium caseinate, the same cross-linking patterns were observed as in the case of laccase A (Fig. 1c and d). The cross-linking was most pronounced when laccase B was used together with FA and cross-linking reaction was carried out at 45 °C (Fig. 1d, lanes 5 and 6). Although no protease activity was detected in the laccase B preparation, one fragmentation band was, however, observed in the samples treated by the high dosage of laccase B at RT (Fig. 1c, lanes 4 and 6) but with a lower intensity than in the experiments where laccase A was used. In the case of laccase A this fragmentation is most probably due to protease side activity present in the preparation (Table 1). For laccase B the minor protein fragmentation could also be due to

the radical-catalyzed reactions. Highly reactive free radicals formed upon the oxidation by laccase can easily undergo non-enzymatic reactions leading to protein fragmentation as well as protein cross-linking (Claus, 2003; Thurston, 1994) and other protein modifying reactions (Steffensen, Andersen, Degn, & Nielsen, 2008). Protein fragmentation due to laccase treatment has been reported for chicken-breast myofibril proteins by Lantto, Puolanne, Kalkkinen, Buchert, and Autio (2005) and for wheat flour doughs by Selinheimo, Kruus, Buchert, Hopia, and Autio (2006).

Laccases have generally been distinguished from tyrosinases because of their inability to oxidize tyrosine (Mayer, 1987). However, Mattinen et al. (2005) showed that *T. hirsuta* laccase is capable of catalyzing the formation of a covalent bond between two aromatic carbons of tyrosine residues in small peptides. Protein conformation, i.e., the accessibility of reactive amino acid residues is one of the main factors determining the extent of protein cross-linking (Mattinen et al., 2006) by enzymes. Proteins are poor substrates for laccase and therefore cross-linking of proteins by laccase is limited, particularly when a mediator compound is not present. The results obtained show that to achieve cross-linking of caseins directly by laccase without using a mediator, a high enzyme dosage and optimal treatment temperature are essential. Selinheimo et al.

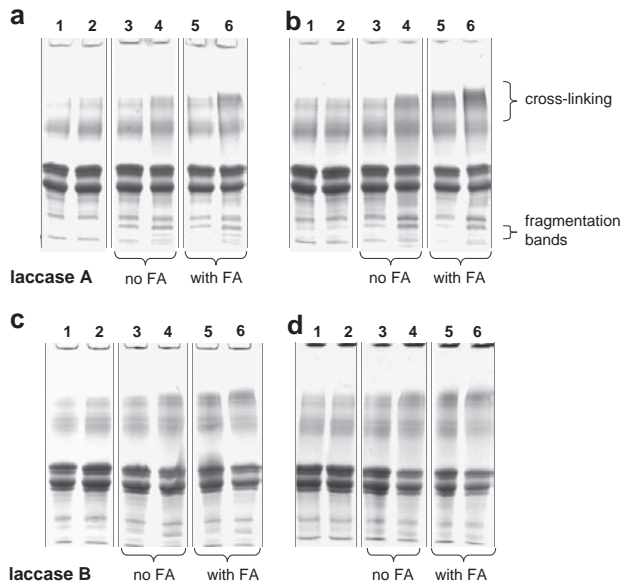


Fig. 1. SDS-PAGE analysis of freeze-dried sodium caseinate gels pre-treated for 2 h by: (a) laccase A at room temperature (RT); (b) laccase A at 45 °C; (c) laccase B at RT; (d) laccase B at 45 °C. Lane 1, control without both laccase and ferulic acid (FA); lane 2, control without laccase but with 2.5 mM FA; lane 3, 2.5 nkat g⁻¹ laccase; lane 4, 25 nkat g⁻¹ laccase; lane 5, 2.5 nkat g⁻¹ laccase + 2.5 mM FA and lane 6, 25 nkat g⁻¹ laccase + 2.5 mM FA.

(2008) also reported that at high dosages, protein cross-linking could be achieved by laccase without a mediator. Steffensen et al. (2008) recently reported that at a low laccase dosage, no cross-linking detectable by SDS-PAGE was achieved in α -casein that was treated by laccase alone but minute amount of dityrosine formation was observed by HPLC. It is clear that presence of an auxiliary or a mediator compound, FA in this case, markedly enhances the formation of high molecular weight reaction products. The efficiency of FA as a mediator in enhancing the protein modification by laccase catalysis has also been reported by other authors (Lantto et al., 2004; Selinheimo et al., 2008; Steffensen et al., 2008).

SEC was used to elucidate the formation of casein dimers, trimers and/or polymers during the gel formation in the presence of laccase and FA. The treatment was carried at 45 °C by laccase A with FA present. The fate of FA was also followed by SEC (Fig. 2). Freeze-dried caseinate gels were incubated in the presence of urea, CHAPS and DTT to achieve complete dissociation of non-covalent bonds and disulfide bridges enabling detection of covalent bonds formed as a result of the laccase action. Peaks for sodium caseinate gel containing FA alone (no laccase) were used as reference. The peak eluting at 15 mL was identified as the casein monomer peak according to Bönisch et al. (2004). FA was found to elute after a retention volume of ~25 mL. Peak eluting at ~23 mL is due to DTT. The formation of casein dimers and trimers by reaction of 2.5 nkat g⁻¹ of laccase together with FA can be seen in Fig. 2 (dotted line). Higher enzyme dosage led also to formation of some casein polymers (Fig. 2, black solid line). Cross-linking efficiency clearly increased as a function of enzyme dosage. SEC analysis revealed the reactivity of FA during the laccase reaction. It is obvious that the FA peak that is shown in the control sample (Fig. 2, grey solid line) completely disappeared already in the presence of the lower laccase dosage. Separate experiments performed in the same conditions to identify the possible FA-FA polymers showed that FA-FA products were eluted in the range of 18–25 mL of retention volume (data not shown) confirming that they do not appear close to the casein monomer or oligomer peaks. It could be deduced that FA molecules were totally bonded into the casein network or alternatively FA-polymerization products were formed which cannot be detected in the conditions used. Steffensen et al. (2008) have recently shown that in the presence of α -casein, more than half of the FA monomers were oxidized into FA dimers by laccase. They claimed that the rest of

the FA was either polymerized, and escaped detection, or were incorporated into the protein matrix (Steffensen et al., 2008). Mattinen et al. (2005) reported that laccase-catalyzed reactions on tyrosine and tyrosine-containing peptides in the presence of FA are rather complex as the enzyme oxidizes FA and tyrosine residues simultaneously. Radicals generated due to enzymatic and non-enzymatic reactions can further react with each other in many different ways leading to various reaction products (Carunchio, Crescenzi, Girelli, Messina, & Tarolla, 2001). It might even be possible that FA monomers are first dimerized and then enzymatically linked into the peptide polymers (Mattinen et al., 2005).

It was observed by both SDS-PAGE and SEC that cross-linking was not very extensive and high amounts of casein monomers remained intact. The cross-linking efficiency of laccase could be increased by e.g. kinetic control of mediator addition. FA is continuously oxidized by laccase and the reaction products either form dimers with other phenoxy radicals or oxidize amino acid residues as stated above. In such a system, FA can also be considered as a competing substrate against protein molecules when present in large amounts. With high FA concentrations it is more probable that the oxidized phenoxy radicals couple to another instead of oxidizing the protein. It has been reported that kinetic control of FA mediated reactions of tyrosine with peroxide is crucial and reaction preference could be changed from FA-FA to tyrosine-FA by keeping FA concentration controlled during the radical reactions (Oudgneog et al., 2001). In this regard, concentration of FA to protein ratio was kept rather small in our study (1:100, w/w). The reaction efficiency could have been probably increased by adding FA in several portions during the course of reaction. Kinetic studies would also be needed to determine the optimum concentration of the mediator that could preferentially lead to protein oligomerisation rather than mediator-mediator coupling.

3.3. Effect of laccase and FA on large deformation properties

Laccase-treated caseinate solutions were chemically acidified by GDL (1.13%) at 25 °C and the texture analysis was carried out after 22 h. The starting pH of the sodium caseinate solution after overnight mixing was 6.8 and final pH of the gels after 22 h incubation at 25 °C was around 4.2. During acidification with GDL, the final pH of the system depends on the amount of GDL added (O'Kennedy, Mounsey, Murphy, Duggan, & Kelly, 2006). In this study, sodium caseinate concentration (5%, w/w), acidification temperature (25 °C) and GDL concentration (1.13% (w/w)) were kept constant for all samples.

In the large deformation measurements, force at the rupture point of the caseinate gels was taken as a measure of gel firmness. Effect of laccase A and laccase B on the firmness of caseinate gels are shown in Fig. 3a and b, respectively. It was observed that firmness of the gels treated with laccase A in the absence of FA were not significantly different from the laccase-free control regardless of the enzyme dosage or pre-treatment temperature used (Fig. 3a). When similar gels were prepared by using laccase B (samples without FA), the gels had significantly ($p < 0.05$) higher force at rupture point values compared with the enzyme-free control except the low-dosage sample pre-treated at room temperature (Fig. 3b). Thus the slight protein cross-linking obtained even without a mediator could be visualized in stronger gel. The significant difference between force at rupture values of laccase A and laccase B treated samples (25 nkat g⁻¹ no FA) seems to be due to presence of proteolytic activity in laccase A. Trace protease activity detected in the laccase A preparation probably altered the structure of the gel counteracting the effect of cross-links formed by laccase. Lantto et al. (2005) investigated the effect of different *T. hirsuta* laccase dosages on gel formation of chicken-breast myofibril protein gels. The results showed that laccase treatment at low

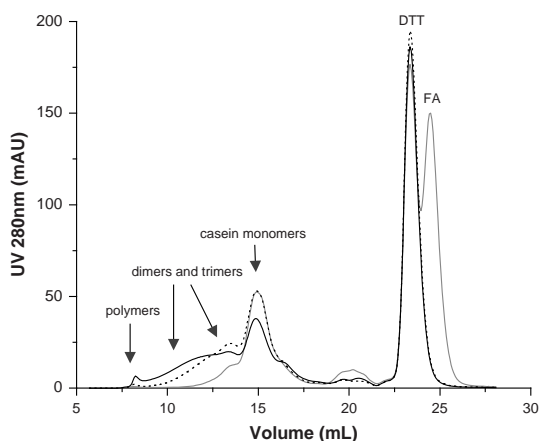


Fig. 2. Size exclusion chromatograms for freeze-dried sodium caseinate gels containing: grey line, control + 2.5 mM ferulic acid (FA); dashed line, 2.5 nkat g⁻¹ laccase A + 2.5 mM FA; black line, 25 nkat g⁻¹ laccase A + 2.5 mM FA.

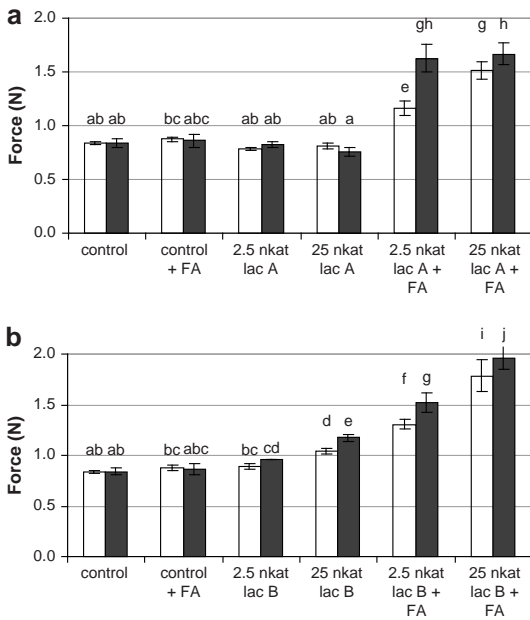


Fig. 3. Firmness (force at rupture point) of sodium caseinate gels acidified for 22 h at 25 °C. Before acidification, sodium caseinate solutions were pre-treated for 2 h at either room temperature (open histogram bars) or 45 °C (closed histogram bars), with and without ferulic acid (FA) and with (panel a) 2.5 and 25 nkat g^{-1} laccase A, or (panel b) laccase B. Values are means and the vertical bars are the standard deviations. Columns with different letters are significantly different at $p < 0.05$.

dosages was not effective to enhance gel formation while there was a slight improvement when an intermediate dosage was used. Further increase of the laccase dosage was shown to result in significantly reduced gel strength most probably due to protein fragmentation. High laccase dosage was also reported to cause softening of wheat bread dough by Selinheimo et al. (2006). In both studies, it was reported that the negative effect of laccase on texture was not due to any protease activity but due to the radical-induced mechanism of laccase. Radical-induced fragmentation can also be a reason why firm gels were not formed in our system.

When laccase treatment was applied together with FA, the firmness of caseinate gels was significantly ($p < 0.05$) higher than the control for all laccase dosages and pre-treatment temperatures (Fig. 3). The effect was more pronounced with laccase B (Fig. 3b). The effect increased as a function of the laccase dosage and pre-treatment temperature for both laccase preparations. Increased enzyme dosage led to the formation of more covalent bonds (Fig. 1) resulting in firmer gels. The bond energy for covalent bonds is a factor of 20–200 higher than for non-covalent bonds (Walstra, 2003). Thus, the force necessary to break strands in which the particles are covalently bound to each other is higher than for non-covalent interactions (Lakemond & van Vliet, 2008a). The firmness of the gels pre-treated at room temperature due to more effective cross-linking at high temperature detected by SDS-PAGE (Fig. 1b and d). In all cases the polymerization was far from complete. Despite of this, the effect of laccase treatment in the presence of FA on gel firmness was significant. Thus only limited cross-linking is required to change the textural properties of acidified sodium caseinate gels.

After incubation for 22 h at 25 °C the gels were stored at 4 °C for 6 days. Laccase was not deactivated before storage as the aim was to monitor potential action of laccase at the low storage temperature. No syneresis was observed in any of the samples at the end of storage. It was observed that after the storage, firmness of the gels with high laccase dosage (25 nkat g^{-1}) together with FA was increased significantly ($p < 0.05$) while there was no change in laccase-free control gel (data not shown). It could be deduced that laccase was able to act at 4 °C on aggregated casein molecules or alternatively, laccase-catalyzed radicals were able to further react at this temperature. Myllärinen et al. (2007) reported that no increase in gel strength of transglutaminase-cross-linked caseinate gels was observed upon storage at 4 °C. The acidic pH of the gels at that stage might help laccase retain its activity and even work while this cannot be the case for TG which has an optimum pH range 5–8 and loses most of its activity around pH 4 (Yokoyama, Nio, & Kikuchi, 2004).

3.4. Effect of laccase and FA on small deformation properties

The gel formation of laccase-treated sodium caseinate solutions was followed by small deformation oscillatory measurements during acidification. Rheological measurements were started 20 min after the addition of GDL (1.13%) to the samples. Storage modulus (G') of the samples was monitored at the acidification temperature of 25 °C for 5 h. The final G' refers to the G' value attained after 5 h from GDL addition. The gelation time, i.e., onset of gelation, and the gelation pH was considered as the time and pH at which G' became ≥ 1 Pa.

The changes in G' with time after GDL addition for the laccase-treated (2.5 nkat g^{-1} and 25 nkat g^{-1}) samples are shown in Fig. 4. Only the samples that were laccase-treated at 45 °C were analyzed. For all the samples, G' vs. time curves had similar shapes with a rapid increase in G' after gelation point and reach of a plateau phase in which G' changes only very slowly. Laccase A or B alone (without FA) did not result in increased final G' (Fig. 4a and c). However, when laccase was used together with FA, increase in final G' compared with laccase-free control gels could be observed for both preparations (Fig. 4b and d). This was in accordance with the large deformation measurements which indicated formation of stiffer gels in the presence of both laccase and FA in the system. Laccase A and laccase B had slightly different gelation profiles depending on the laccase dosage. In Fig. 4a, it was shown that when sodium caseinate was pre-treated with high dosage of laccase A (without FA) at 45 °C, it gained a lower final G' than the laccase-free control gel. The weakening of the gel strength could be attributed to the detectable protease activity in laccase A preparation, since no reduction in final G' was observed when laccase B was used at the same dosage without FA (Fig. 4c). Furthermore, when laccase A was used together with FA, final G' of the samples for both laccase dosages were similar, i.e., no increase in G' with increased laccase dosage. The same result was observed in force at rupture point values (Fig. 3a) and attributed to high protease load in the reaction when high laccase A dosage was used. Weakening of the bonds could be seen in both small and large deformation analysis.

After the final G' was attained, each sample was subjected to a frequency sweep. Graphs of $\log G'$ vs \log frequency were plotted for each sample. All samples produced straight lines within the given frequency interval with a similar slope of around 0.18–0.20 indicating no distinct differences regarding the dependency of G' on frequency (data not shown). Slope of $\log G'$ vs \log frequency curve has been used by many authors as an indication of the state of the overall bonds in acidified milk systems (Anema et al., 2005; Lakemond & van Vliet, 2008b; Lucey, Tamehana, Singh, & Munro, 2000). Anema et al. (2005) also reported that although final gel strength

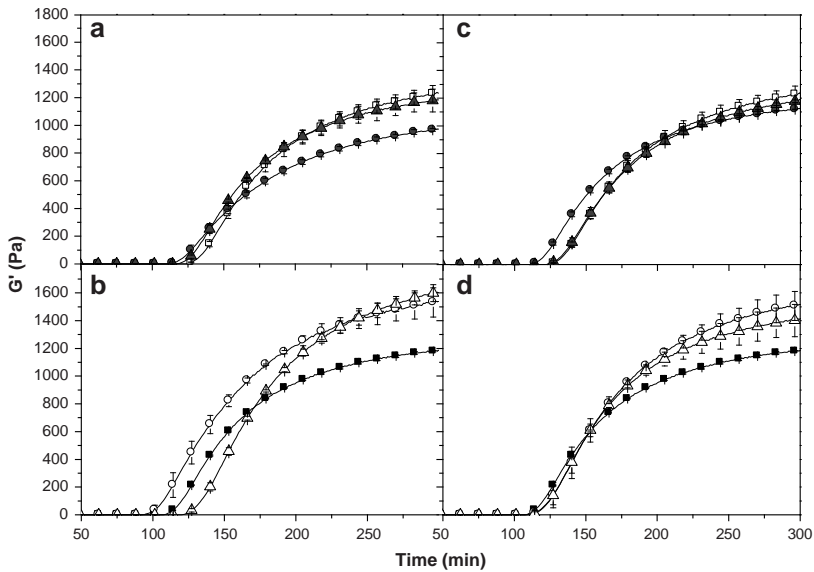


Fig. 4. Development of the storage modulus (G') during gelation. Before acidification, sodium caseinate solutions were pre-treated for 2 h at 45 °C with: (a) laccase A; (b) laccase A + ferulic acid (FA); (c) laccase B; (d) laccase B + FA. In panels (a) and (c) the symbols represent: (□), control without both laccase and FA; (▲), 2.5 nkat g⁻¹ laccase; (●), 25 nkat g⁻¹ laccase. In panels (b) and (d) the symbols represent: (■), control + FA; (△), 2.5 nkat g⁻¹ laccase + FA; (○), 25 nkat g⁻¹ laccase + FA. Vertical bars are the standard deviations at each data point.

was significantly altered when TG was used in acid skim milk gels, the frequency dependence of G' for the final gels was not different from the TG-free control. Based on Lakemond and van Vliet (2008b), we can conclude that although final G' is altered due to introduction of new covalent bonds, there are no indications of differences in formation of structural rearrangements in the final gels.

Slight differences like 10–20 min in gelation time ($G' > 1$ Pa) was observed for some of the samples (Fig. 4). As the amount of GDL and the acidification temperature were constant, such differences were likely to be due to the enzyme reaction and/or FA. pH profiles of the samples after GDL addition revealed that addition of 2.5 mM FA into the sodium caseinate solution resulted in slight pH drop at the time 0 (before GDL addition) and led to lower pH values in other time points compared with the FA-free control solution (Fig. 5). But, the gelation pH was not affected as shown on Fig. 5. Gelation pH of the enzyme-treated samples were also determined to be pH 5.18 ± 0.03 (data not shown). It can be concluded that, effect of FA and slight changes in water pH resulted in slight shifts in gelation points of the samples. O'Kennedy et al. (2006) suggested that cross-linking enzymes can be a factor that would speed up the connectivity of caseins in the initial phase, in addition to loss of electrostatic repulsions and lead to an earlier onset of gelation at a higher gelation pH. Decrease in gelation time was observed by TG treatment of sodium caseinate (Myllärinen et al., 2007; Schorsch et al., 2000) and also in sugar beet pectin gels by laccase (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003). In our system, modification was probably not enough to make a significant effect on the gelation time and pH. Furthermore, the additional effect of FA on solution pH made it difficult to interpret the effect of the enzymatic modification.

In accordance with the large deformation results, it was observed that already a small increase in the extent of cross-linking was enough to increase the final G' of the gels. Roefs and van Vliet (1990) reported that the storage modulus of acidified casein gels is dependent on the number and strength of bonds between casein particles. It is expected that the final G' will be higher when relatively more covalent bonds

are present (Lakemond & van Vliet, 2008a). In the case of transglutaminase, it has been reported that, a small increase in the number of covalent cross-links results in increased final G' (Anema et al., 2005), firmness (Færgemund & Qvist, 1997; Færgemund, Sorenson, Jørgensen, Budolfson, & Qvist, 1999), and breaking strain (Lauber et al., 2000) of acid milk gels. Bönisch et al. (2007) showed that TG-induced protein polymerization in the range of 10–30% was required to improve the rheological properties of yoghurt gels.

Acidification of non-enzyme-treated caseins is known to induce mainly physical bond formation. A three dimensional gel network is formed due to van der Waals forces, loss of steric stabilization, hydrophobic and electrostatic interactions as well as hydrogen

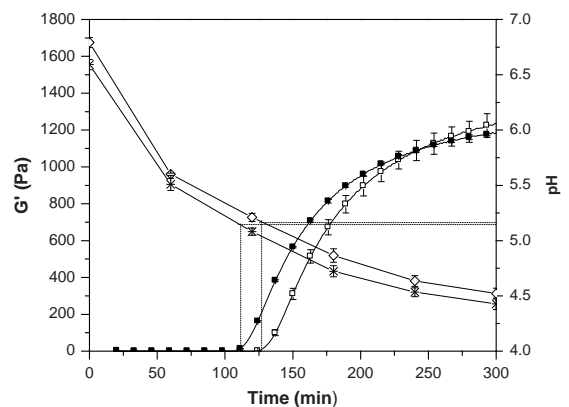


Fig. 5. Storage modulus (G') and pH as a function of time during acidification of control sodium caseinate gel with/without ferulic acid (FA) after addition of GDL (1.13%) at 25 °C. G' control without FA (□); G' control + FA (■); pH control without FA (●); pH control + FA (*). Vertical bars are the standard deviations at each data point.

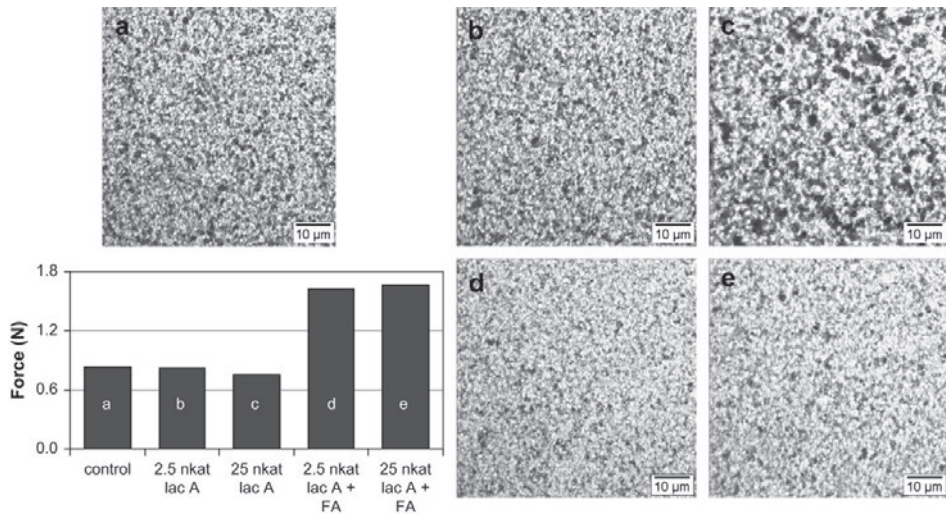


Fig. 6. Confocal laser scanning microscopy images of sodium caseinate gels acidified with GDL (1.13%) at 25 °C for 22 h. Before acidification, sodium caseinate solutions were pre-treated by laccase A for 2 h at 45 °C with the additions: (a) control without both laccase and ferulic acid (FA); (b) 2.5 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. Protein matrix is white, and aqueous parts or pores are dark. Firmness (force at rupture point) of laccase A treated gels was also shown for correlation with micrographs.

bonds (Jaros et al., 2006). In this study, the creation of covalent cross-links between casein proteins by using *T. hirsuta* laccase, and FA was found to increase gel firmness according to both small and large deformation measurements. Possible promotion of other interactions, e.g., non-covalent interactions as well as covalent cross-links was not investigated in this study. It is very likely that enzyme-induced modification of proteins might also alter their surface tension and hydrophobicity resulting in modified functional properties as suggested by Steffensen et al. (2008).

3.5. Effect of laccase and FA on gel microstructure

The microstructure of sodium caseinate gels pre-treated at 45 °C for 2 h with laccase A with or without FA was also analyzed. Micrographs and the large deformation graphs of the related samples are shown in Fig. 6. Protein particles appear as white areas while aqueous phase or pores are dark.

Microstructural properties of the gels obtained were mostly similar to each other, i.e., the structure was homogenous with small aggregates linked together. Sodium caseinate gels are usually classified as particle gels and the structure of the particles determine the gel properties (van Vliet, Lakemond, & Visschers, 2004). When caseinate gels were treated with laccase A without FA, the effect was not significant with the dosage of 2.5 nkat g⁻¹ (Fig. 6b) while a clear increase in pore size compared with the laccase-free control (Fig. 6a) was observed for the sample treated with 25 nkat g⁻¹ of laccase (Fig. 6c). Although the force at rupture point for the same sample was not significantly different from the control gel, small deformation measurements revealed a decreased final *G'* (Fig. 4a) indicating the weakening of the gel structure when laccase A was used alone. This was attributed to the trace proteolytic activity present in laccase A leading to a coarser microstructure. A clear decrease in the pore density and a finer network was observed at both laccase dosages when laccase A was used together with FA (Fig. 6d and e) indicating formation of stronger gels, which is very much in accordance with small and large deformation measurements. As there are no other reports on effect of laccase on acidified sodium caseinate gel microstructure, it was compared with the effect of TG. TG-treated

casein gels were reported to have a homogeneous microstructure with finer network and smaller pores compared with non-TG-treated gels (Færgemand & Qvist, 1997; Myllärinen et al., 2007; Schorsch et al., 2000). Covalent bonds induced by TG treatment stabilized the structure by forming more interactions and stronger cross-links (Myllärinen et al., 2007). The same phenomenon is valid for laccase only if it is used with an auxiliary substance and in relatively high dosages compared with TG.

4. Conclusions

These results show for the first time the effects of *T. hirsuta* laccase on gel formation and structure of acidified sodium caseinate gels. Under the given conditions and with the enzyme dosages used, it was observed that caseins were both cross-linked and fragmented by the action of laccase. The extent of cross-linking was increased by increasing laccase dosage, increasing temperature and presence of FA. Addition of FA to the laccase reactions enhanced cross-linking of caseins leading to significantly firmer sodium caseinate gels with higher final *G'* values and finer microstructure compared with the situation where laccase was used alone. Two different laccase preparations were used to evaluate the effect of protease side activity that might be present in enzyme preparations. Laccase preparation containing detectable amount of protease resulted in weaker sodium caseinate gels compared with the gels treated by laccase preparation which was devoid of any detectable protease activity. The main reason of the protein fragmentation is suggested to be the trace amount of protease activity in the laccase preparation. Extent of possible protein fragmentation due to radical-induced reaction mechanism of laccase needs further elucidation.

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PUBLICATION III

**Effect of *Trichoderma reesei*
tyrosinase on rheology and
microstructure of acidified
milk gels**

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Effect of *Trichoderma reesei* tyrosinase on rheology and microstructure of acidified milk gels

D. Ercili Cura*, M. Lille, R. Partanen, K. Kruus, J. Buchert, R. Lantto

VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

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ABSTRACT

The aim of this work was to study the potential of tyrosinase enzymes in structural engineering of acid-induced milk protein gels. Fat free raw milk, heated milk or a sodium caseinate solution were treated with tyrosinases from *Trichoderma reesei* (TrTyr) and *Agaricus bisporus* (AbTyr) and the reference enzyme transglutaminase (TG) prior to acid-induced gelation. TrTyr treatment increased the firmness of raw milk and sodium caseinate gels, but not that of heated milk gels, even though protein cross-linking was detected in heated milk. AbTyr did not cross-link proteins in any of the studied milk protein systems. TG was superior to TrTyr in gels prepared of heated milk. In acidified heated milk and sodium caseinate, TrTyr and TG treatment resulted in a decrease of the pore size. Scanning electron microscopy revealed more extensive particle interactions in the heated milk gels with TG than with TrTyr.

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1. Introduction

Milk gels are mainly stabilized by weak non-covalent interactions. The introduction of covalent bonds to the system leads to alterations in the structural properties of the gel. This has been shown by comparing the mechanical properties of milk gels made of raw and heat-treated milk. Heat-induced denaturation of whey proteins and subsequent interaction with caseins via thiol/disulfide bridges increase the strength of acidified gels (Anema, Lauber, Lee, Henle, & Klostermeyer, 2005; Lucey, Tamehana, Singh, & Munro, 1998, 2001; Vasbinder & de Kruif, 2003). Incorporating additional covalent bonds by enzymes, such as transglutaminase (TG), is an efficient way of modifying the structural properties of milk protein systems. The mode of action and exploitation of TG in milk products has been extensively studied and well reviewed by Jaros, Partschefeld, Henle, and Rohm (2006). In addition to TG, which is a transferase, oxidative enzymes such as laccase and tyrosinase have also shown ability to induce covalent cross-links between food proteins as reviewed by Buchert et al. (2007). We recently reported that the firmness of acidified sodium caseinate gels could be increased by cross-linking the proteins with laccase before acidification (Ercili Cura et al., 2009). Hitherto, it has been unknown whether also tyrosinase could be used as a structure modifier of acid-induced milk protein gels.

Tyrosinases (EC 1.14.18.1) are mono-oxygenases which are widely distributed in nature (Claus & Decker, 2006). Likewise laccase, tyrosinase is capable of oxidizing tyrosine residues in proteins leading to intermolecular protein cross-linking, however, through a different reaction mechanism. Tyrosinases catalyse two distinct oxidative reactions, *ortho*-hydroxylation by monophenolase (creosolase) and oxidation by diphenolase (catecholase) activity (Fig. 1) with subsequent production of highly reactive *ortho*-quinones. Oxygen is the co-substrate in both of these reactions (Solomon, Sundaram, & Machonkin, 1996). In food 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 sulphhydryl and amino groups resulting in the formation of tyrosine–tyrosine, tyrosine–cysteine and tyrosine–lysine cross-links (Burzio, Burzio, Pardo, & Burzio, 2000; Ito, Kato, Shinpo, & Fujita, 1984; Kato, Ito, & Fujita, 1986; Matheis & Whitaker, 1984).

Typically the main focus in tyrosinase research has been in inhibition of the endogenous plant tyrosinase (also called polyphenol oxidase) activity to control browning. Exploitation of tyrosinase in food structure modification is a relatively new area. The cross-linking ability of a tyrosinase from the filamentous fungus *Trichoderma reesei* has been studied in various foods such as meat (Lantto, Puolanne, Kruus, Buchert, & Autio, 2007), wheat bread (Selinheimo, Autio, Kruus, & Buchert, 2007b) and model protein systems including dairy proteins such as bovine serum albumin (BSA) and β -casein (Mattinen, Lantto, Selinheimo, Kruus, & Buchert, 2008; Monogioudi et al., 2009) and α -casein (Selinheimo et al., 2007a). Cross-linking of α -lactalbumin (as such) and β -lactoglobulin (in the

* Corresponding author. Tel.: +358 40 015 7616; fax: +358 20 722 7071.
E-mail address: dilek.ercili@vtt.fi (D. Ercili Cura).

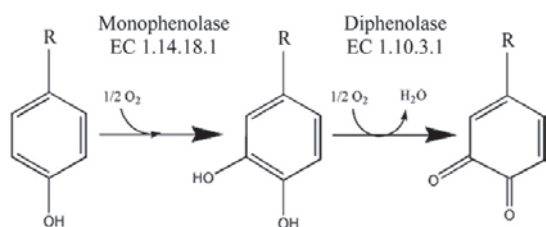


Fig. 1. Oxidation of a monophenol (protein-bound tyrosine) to an *o*-diphenol and an *o*-quinone by tyrosinase (modified from Claus & Decker, 2006; Solomon et al., 1996).

presence of caffeic acid) by *Agaricus bisporus* tyrosinase has been reported by Thalmann and Lötzbeyer (2002). The tyrosinase from the fungus *Pycnoporus sanguineus* has been shown to cross-link caseins (Halaoui, 2005). Selinheimo et al. (2007a) showed that tyrosinase from *P. sanguineus* and *T. reesei* could cross-link α -casein while tyrosinase from *A. bisporus* and plant tyrosinases were able to create cross-links only in the presence of an auxiliary compound. 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, 2008). Moreover, tyrosinases were also reported to induce formation of protein–oligosaccharide conjugates (Selinheimo, Lampila, Mattinen, & Buchert, 2008) and grafting of peptides onto polysaccharides (Aberg, Chen, Olumide, Raghavan, & Payne, 2004). Although very potent enzymes for protein cross-linking and structure modification, tyrosinases are not yet commercially available for large scale applications (Buchert et al., 2007).

Caseins are the main structural component of dairy products, such as yoghurt and cheese. Therefore, enzymatic tailoring of the interactions between casein molecules is expected to alter the structural properties of the milk protein-based systems. To our knowledge, there are only a few reports (Ercili Cura et al., 2009; Onwulata & Tomasula, 2008; Steffensen, Andersen, Degn, & Nielsen, 2008) focused on the utilization of oxidative enzymes in structure modification of dairy systems. The aim of this work was to elucidate the gelation of milk proteins upon modification by two tyrosinases of different origin, i.e., *T. reesei* tyrosinase and *A. bisporus* tyrosinase. *Streptomyces mobaraense* TG was used as a reference enzyme. In addition to sodium caseinate, the milk protein source used in our previous laccase paper (Ercili Cura et al., 2009), also raw and heated skim milk were included as raw materials in the tyrosinase work. Since TG was reported to induce only limited cross-linking in unheated or mildly heated (<80 °C) milk protein systems (De Jong, Wijngaards, & Koppelman, 2003), comparison of the effects of *T. reesei* tyrosinase and TG in raw and heated (5 min at 90 °C) milk was carried out. The structural properties of chemically acidified milk and sodium caseinate gels were analyzed by rheological and microscopical techniques.

2. Materials and methods

2.1. Enzymes and enzyme activity assays

T. reesei tyrosinase (TrTyr) was produced and purified according to Selinheimo et al. (2006). *A. bisporus* tyrosinase (AbTyr) was purchased from Fluka Biochemica (Taufkirchen, Germany) and dissolved (5 mg mL⁻¹) in 0.1 M sodium phosphate buffer (pH 7.0) before use. Tyrosinase activity was assayed using 15 mM L-DOPA (Sigma–Aldrich, St. Louis, MO, USA) as substrate at pH 7.0 and room temperature according to Robb (1984). Activa[®] WM transglutaminase (TG) preparation was supplied by Vesaniti Oy (Helsinki, Finland) and further purified as described in Lantto,

Puolanne, Kalkkinen, Buchert, and Autio (2005). The activity of the TG preparation was assayed by the colorimetric hydroxamate method (Folk, 1970) using 0.03 M N-carbobenzoxy-L-glutaminylglycine (Sigma–Aldrich) as substrate at pH 6.0. The enzyme activities measured for TrTyr, AbTyr and TG were 1600 nkat mL⁻¹, 500 nkat mL⁻¹, and 8700 nkat mL⁻¹, respectively.

2.2. Gel preparation

Raw skim milk (3.61% protein, 0.08% fat, 5.06% lactose, 9.34% of dry matter) was supplied by Valio Ltd (Helsinki, Finland). Sodium caseinate powder (EM 7, protein content 91%) was from DMV International (Veghel, the Netherlands). When needed, raw skim milk was heated at 90 °C for 5 min in a water bath and cooled by immersion in ice. Sodium caseinate solution (3%, w/w) was prepared as described in Ercili Cura et al. (2009). Raw milk, heated milk and sodium caseinate samples were tempered to 40 °C prior to the addition of an enzyme. The enzyme dosages used were 100 nkat g⁻¹ protein for AbTyr and TrTyr, and 25 nkat g⁻¹ protein for TG. No enzyme was added to the control sample. All samples were incubated at 40 °C for 1 h. After the enzyme treatment, samples were tempered to 30 °C and acidified by addition of 1.2% and 0.45% D-(+)-gluconic acid δ -lactone (GDL) (Sigma–Aldrich) for milk and sodium caseinate samples, respectively. Gel formation was carried out at 30 °C for 20 h. The final pH of the samples was about 4.6.

2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis

SDS–PAGE analysis was performed under reducing conditions as described in Ercili Cura et al. (2009). Unlike in the previous paper, freeze-dried gels were dissolved directly in the SDS–PAGE loading buffer (Tris–HCl with β -mercaptoethanol and SDS) to a concentration of 2 mg mL⁻¹ to achieve complete solubilization of the cross-linked protein aggregates. Ready-made 10–20% Tris–HCl polyacrylamide gels (Bio-Rad Criterion[™] Precast gel, Hercules, CA, USA) and pre-stained molecular weight markers (Pre-stained SDS–PAGE standards, broad range, Bio-Rad) were used.

2.4. Rheological characterization

Small amplitude oscillatory measurements were performed by using a stress-controlled rheometer (StressTech, Reologica Instruments AB, Lund, Sweden) as described in Ercili Cura et al. (2009) with modifications. After GDL addition, samples were stirred vigorously for 5 min and placed into the concentric cylinder (CC 25) measuring system. Silicon oil was added on the sample surface to prevent evaporation. Samples were oscillated at a frequency of 0.1 Hz and at a strain of 0.01 which is in the linear viscoelastic region of the milk gels. Storage modulus (G') and loss tangent ($\tan \delta = G''/G'$) of the samples were monitored during gel formation. Results are mean values of two repetitions. The final G' and the final $\tan \delta$ refer to the G' and $\tan \delta$ values attained after 20 h of GDL addition. The gelation time and the gelation pH were considered as the time and pH at which G' became ≥ 0.1 Pa for raw milk and $G' \geq 1$ Pa for heated milk and sodium caseinate gels.

Large deformation measurements were performed using a TA-HDi Texture Analyser (Stable Microsystems Ltd., Godalming, UK) by the method described previously (Ercili Cura et al., 2009). The area under the force-deformation curve (up to a depth of 8 mm) was taken as a measure of gel firmness. Measurements were performed on four individual gels for each sample and results were analyzed by one-way analysis of variance (ANOVA) followed by a comparison of mean values test. Values were considered to be significantly different at $p < 0.05$.

2.5. Confocal laser scanning microscopy

The microstructure of the samples was examined with a confocal laser scanning microscope (CLSM) (Bio-Rad, Hemel Hempstead Herts, UK) equipped with helium–neon laser emitting at 543 nm. The fluorescent dye Rhodamine B (Merck, Darmstadt, Germany) was excited at 543 nm and the emitted light was recorded with an E570LP emission filter. Samples were prepared by mixing 5 mL of the milk or sodium caseinate solution with 25 μ L of the Rhodamine B solution (0.1%, w/w) and were acidified and examined as such on special object glasses described previously in Ercili Cura et al. (2009).

2.6. Scanning electron microscopy

The morphology of the protein particles forming the milk gels was analyzed by a field-emission scanning electron microscope (SEM; Leo DSM982 Gemini, Leo Electron Microscopy Inc., Germany). After 20 h at 30 °C, the gels were cut into cubes (5 × 5 × 5 mm) and fixed in 2.5% (v/v) glutaraldehyde solution in 0.1 M sodium phosphate buffer (pH 7.0) for 24 h at 4 °C. The gel pieces were rinsed several times with distilled water and dehydrated in a series of aqueous ethanol solutions (from 10 to 100%) for 30 min intervals. Samples were frozen by immersion in liquid nitrogen and freeze dried for 48 h. Freeze-dried gel specimens were crushed in a mortar into a fine powder and mounted on SEM aluminum stubs with double-sided conductive carbon tabs. Images were obtained at a voltage of 1.4 kV.

3. Results

3.1. Protein cross-linking

Protein cross-linking was not observed by AbTyr treatment either in raw or heated milk (Fig. 2a, b: lane 2). TrTyr was able to cross-link milk proteins both in raw and heated milk, which was observed as weakening of the casein bands and formation of high molecular weight protein bands (Fig. 2a, b: lane 3). The extent of cross-linking by TrTyr was similar both in raw and heated milk. Mainly trimers (bands around 75 kDa) and higher oligomers (>150 kDa) were observed as well as protein aggregates at the bottom of the wells. It was observed that mainly β -casein was cross-linked while α - and κ -caseins remained almost intact

(Fig. 2a, b: lane 3). TG did not cross-link proteins in raw milk (Fig. 2a: lane 4). This was an expected result as TG is reported to induce only minor cross-linking of unheated milk proteins due to the presence of a heat labile low molecular weight inhibitor of TG in bovine milk serum (De Jong et al., 2003). Recently it has been reported that addition of reducing agents such as glutathione to raw milk can prevent the inhibition of TG (Bönisch, Lauber, & Kulozik, 2007). In heated milk, TG treatment resulted in formation of casein dimers and trimers (bands around 50–75 kDa), and larger oligomers (>150 kDa) as well as aggregates observed at the bottom of the well (Fig. 2b: lane 4). According to SDS-PAGE (Fig. 2a, b), cross-linking of α -lactalbumin or β -lactoglobulin was not observed with tyrosinases or TG in either raw or heat-treated milk.

In addition to protein cross-linking in milk, cross-linking of sodium caseinate was also analyzed. Similarly as in milk, AbTyr had no effect on proteins in sodium caseinate solution (Fig. 2c: lane 2). On the other hand, TrTyr and TG resulted in extensive protein cross-linking (Fig. 2c: lanes 3 and 4, respectively). As in milk, in sodium caseinate TrTyr and TG resulted in formation of protein oligomers and larger polymers. Mainly β -casein was cross-linked with both enzymes. Weakening of band intensity was also observed for α -caseins while κ -casein remained almost intact both in TrTyr and TG treatments.

3.2. Gel formation

Acid gels prepared of raw milk showed a slightly different gel formation profile than those prepared of heated milk (Fig. 3a, b). The gelation pH was 5.4 for heated milk and about pH 4.9 for raw milk. Final G' of the enzyme-free heated milk control gel was 435 Pa while it was 17 Pa for gel prepared of raw milk (Table 1). $\tan \delta$ versus time graphs also showed different profiles between raw and heated milk. In the heated milk control sample, a peak in $\tan \delta$ was observed right after the gelation point while no peak was present in raw milk samples.

The change in pH during acidification was followed for both control and enzyme-treated samples and no difference in pH profiles was observed due to the enzymatic modification. Therefore, the pH profiles of the control gels only were plotted together with G' (Fig. 3). In raw milk, gelation started after 288 min of GDL addition, and the gelation pH was 4.9 (Table 1). TrTyr treatment resulted in almost 1 h earlier gelation time (230 min) and increase in gelation pH to 5.0 (Table 1). TrTyr treatment had a marked effect on the rate of gel formation (the initial slope of the G' versus time curve) and the final G' (Fig. 3a). The final G' of the TrTyr-treated gel was 58 Pa while that of the enzyme-free control was 17 Pa (Table 1). On the other hand, AbTyr and TG treatments resulted in similar gel development curves and final G' values with the enzyme-free control gel. Development of $\tan \delta$ was also consistent with the development of G' . A lower final $\tan \delta$ value was observed for the TrTyr-treated gel than for the control gel (Table 1), indicating a difference in viscoelastic character between the gels.

In heated milk, enzymatic cross-linking prior to GDL addition did not markedly affect either the gelation time or gelation pH during gel formation (Fig. 3b, Table 1). TG treatment led to a firmer gel with considerably higher G' compared with the enzyme-free control and AbTyr- and TrTyr-treated gels (Table 1). Either tyrosinase did not have a positive effect on G' (Fig. 3b). The cross-linking efficiency of TG in heated milk was verified also with SDS-PAGE (Fig. 2b: lane 4). A dosage of 25 nkat TG per g protein resulted in an almost two-times higher final G' compared with the other samples and also final $\tan \delta$ value was lowered from 0.23 for control gel to 0.17 in TG-treated gel (Table 1). Even though TrTyr treatment resulted in protein cross-linking comparable with TG (Fig. 2b: lane 3), there was no effect on the development of G' with TrTyr.

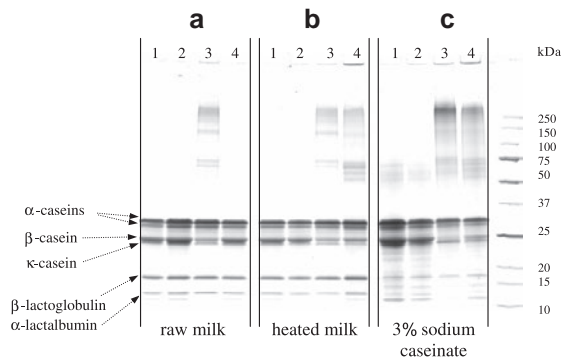


Fig. 2. SDS-PAGE analysis of freeze dried (a) raw milk, (b) heated milk and (c) sodium caseinate gels that were pre-treated by cross-linking enzymes before acidification: lane 1, enzyme-free control; lane 2, substrate + AbTyr; lane 3, substrate + TrTyr; lane 4, substrate + TG. Molecular mass standards (kDa) are labelled on the far right of the gels.

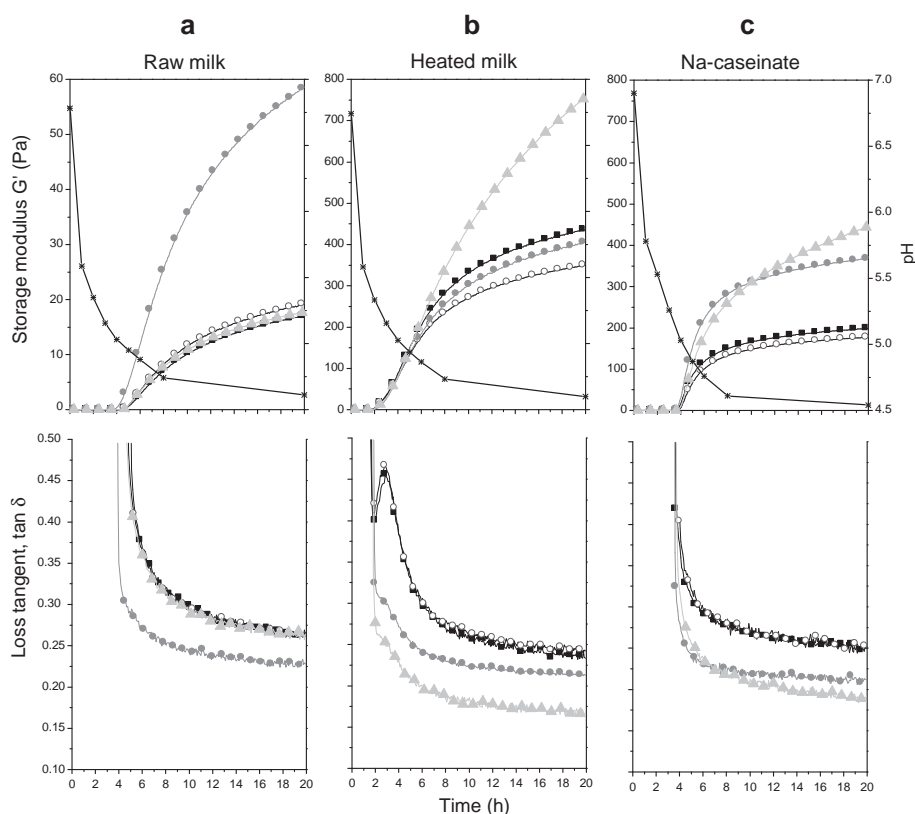


Fig. 3. Storage modulus (G') and loss tangent ($\tan \delta$) of acidified (a) raw milk, (b) heated milk, and (c) sodium caseinate gels. Before acidification, samples were treated with (■) no enzyme (control), (○) AbTyr, (●) TrTyr, or (▲) TG for 1 h at 40 °C. The change in pH during acidification of the control samples is also shown in the upper graphs (✱).

Table 1
Rheological parameters determined by small deformation oscillatory measurements during gelation of enzyme-treated raw milk, heated milk and sodium caseinate solution.^a

	Gelation time (min)	Gelation pH	pH at $\tan \delta$ peak	Final $\tan \delta^b$	Final G' (Pa) ^b
Raw milk					
Control	288	4.9	np ^c	0.26	17
AbTyr	281	4.9	np	0.27	19
TrTyr	230	5.0	np	0.23	58
TG	280	4.9	np	0.26	18
Heated milk					
Control	110	5.4	5.1	0.23	435
AbTyr	110	5.4	5.2	0.24	350
TrTyr	125	5.4	np	0.21	405
TG	115	5.4	np	0.17	755
Sodium caseinate					
Control	224	5.1	np	0.25	199
AbTyr	226	5.1	np	0.25	178
TrTyr	216	5.1	np	0.21	368
TG	226	5.1	np	0.19	446

^a Values represented are means of duplicate measurements with an experimental error lower than 10%.

^b $\tan \delta$ and G' values attained after 20 h incubation at 30 °C.

^c np: no peak.

However, the $\tan \delta$ profile of TrTyr-treated sample was different from that of the enzyme-free control gel. The final value of $\tan \delta$ was lowered to 0.21 in TrTyr-treated gel. Furthermore, the peak in $\tan \delta$ observed for control and AbTyr-treated gels around pH 5.1–5.2 was missing for TrTyr- and TG-treated gels (Fig. 3b, Table 1).

The gel formation of a 3% sodium caseinate solution was studied under the same conditions as those used for milk gels. The range of final G' values for sodium caseinate gels were between those of raw and heated skim milk gels (Fig. 3c, Table 1). AbTyr treatment showed no effect on either development of G' or $\tan \delta$ whereas both TG- and TrTyr-treated sodium caseinate gels attained higher final G' and lower final $\tan \delta$ values than the control gel (Fig. 3c, Table 1).

3.3. Gel texture

The shape of the force-deformation curve was similar in all samples. Firmness of the raw milk and heated milk control gels were measured to be 0.34 N mm and 0.80 N mm, respectively (Fig. 4). Firmness of the raw milk gels was not significantly affected by AbTyr or TG, but firmness of the TrTyr-treated raw milk gel was found to be significantly ($p < 0.05$) higher than that of the enzyme-free control (Fig. 4a). This result is in accordance with the final G' value for the similarly treated gel made of raw milk. TG treatment did not affect the firmness of the raw milk gel. In heated milk, the AbTyr or TrTyr treatments did not affect the gel firmness (Fig. 4b), even though protein cross-linking with TrTyr was on a similar level

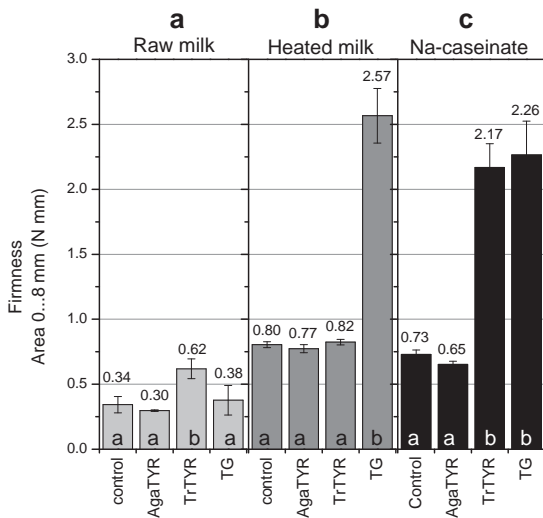


Fig. 4. Firmness (large deformation) of acidified (a) raw milk, (b) heated milk and (c) sodium caseinate gels. Before acidification, samples were treated by AbTYr, TrTYr or TG for 1 h at 40 °C. Vertical bars represent the standard deviations. Statistical analysis was performed separately for each substrate. Columns with different letters within each substrate are significantly different at $p < 0.05$.

with that in raw milk (Fig. 2b). This phenomenon was also observed in the small deformation measurements (Fig. 3b). TG-treated gel was significantly ($p < 0.05$) firmer than the control gel (Fig. 4b). As a result, TG was capable of forming markedly firmer gels than TrTYr of heated milk (Fig. 4b).

Large deformation tests applied to gels prepared of 3% sodium caseinate solution showed that, both TrTYr and TG treatments resulted in significantly higher ($p < 0.05$) gel firmness compared with the control while AbTYr was ineffective (Fig. 4c).

3.4. Gel microstructure by confocal laser scanning microscope

Microstructures were analyzed only for TrTYr- and TG-treated gels as AbTYr did not cross-link milk proteins nor had an effect on the rheological properties of the milk protein gels. In raw milk, control and the enzyme-treated gels showed similar characteristics (Fig. 5). Gel structures were composed of large clusters of protein aggregates and few contact points between clusters. However, it could be said that TrTYr-treated gel had slightly denser structure and more homogenous pores than the other two (Fig. 5). Both enzymes affected the microstructure of the heated milk gels in similar ways (Fig. 5). The gels had a finer and denser structure with more homogenous and smaller pores than the control gel.

Sodium caseinate gels showed very similar microstructures to that of heated milk gels (Fig. 5). Large pores and long strands were observed in the control gel, whereas TrTYr and TG treatments resulted in increased connectivity between the aggregates, a finer network and smaller pores. As reported earlier (Myllärinen, Buchert, & Autio, 2007; Partanen et al., 2008; Schorsch, Carrie, & Norton, 2000), TG-treated caseinate gels had more homogeneous microstructure with smaller pores than non-TG-treated gels.

3.5. Scanning electron microscope analysis of milk gels

SEM was used to identify the structural differences between TrTYr- and TG-treated milk gels (Fig. 6) as resolution and magnification of CLSM was not enough to reveal any differences. SEM

images clearly showed that, TrTYr-treated raw milk gel was composed of considerably smaller casein particles compared with TG-treated gel (Fig. 6a, b). TG-treated gel had very similar particle size to the control raw milk gel (image not shown). In TrTYr- and TG-treated heated milk gels (Fig. 6c, d), size of the particles was considerably smaller compared with the heated milk control gel (image not shown). The main difference between TrTYr- and TG-treated heated milk gels was the extent of inter-particle interactions which could clearly be observed by SEM but not by CLSM. Casein particles were fused into each other forming relatively long protein strands between protein particles or cluster in TG-treated heated milk gel (Fig. 6d). However, TrTYr-treated heated milk gel was composed of individual protein particles aggregated without clear fusion or strand formation (Fig. 6c).

4. Discussion

TrTYr cross-linked caseins in both raw and heated skim milk, whereas AbTYr was ineffective. The two tyrosinases have been reported to have differences in their cross-linking ability (Mattinen et al., 2008; Selinheimo et al., 2007a). Selinheimo et al. (2007a) found that TrTYr is capable of cross-linking α -caseins as such, while AbTYr needs an auxiliary compound for cross-linking. It was hypothesized that the inability of AbTYr to oxidize casein-bound tyrosine might be related to limited accessibility of tyrosine residues to the active site. TrTYr has been reported to cross-link cereal (Selinheimo et al., 2007b) and meat (Lantto et al., 2007) proteins without an auxiliary component supporting the above mentioned observations about the ability of TrTYr for direct oxidation of protein-bound tyrosine residues. In this respect, TrTYr was found to be superior compared with laccase as well. In our earlier publication, we showed that laccase could cross-link caseins and subsequently modify caseinate gel structure only in the presence of a small phenolic compound (Ercili Cura et al., 2009).

Heat treatment at 90 °C led to increased gelation pH (decreased gelation time) and increased final G' of the milk gels compared with raw milk. The formation of soluble and micelle-bound whey protein/k-casein complexes modifies the surface properties of micelles in heated milk causing increased hydrophobicity together with increased apparent pI towards the pI of whey proteins (Guyomarc'h, Law, & Dalgleish, 2003; Guyomarc'h, Nono, Nicolai, & Durand, 2009; Lucey et al., 1998), and increased G' (Lucey et al., 1998). Enzymatic cross-linking prior to GDL addition did not affect the gelation time or pH in heated milk and sodium caseinate, and an increase (about 0.1 units) in gelation pH was observed in raw milk treated by TrTYr. The increase in gelation pH (decrease in gelation time) and the steep increase in G' versus time curve in TrTYr-treated raw milk gel indicates that the extent of cross-linking in raw milk was enough to modify aggregation and re-arrangement behaviour of casein particles during gelation. A decrease in gelation time was reported by TG treatment of micellar casein dispersion (Schorsch et al., 2000) and heated skim milk (Anema et al., 2005). In our study, the extent of cross-linking with TG in heated milk and sodium caseinate was probably not enough to cause a considerable effect on the gelation time and pH (Fig. 3b, c; Table 1).

The occurrence of the $\tan \delta$ peak in the heated milk gel (Fig. 6b) is mainly related to the weakening of the intra-micellar interactions during the solubilization of colloidal calcium phosphate (CCP) below pH 5.8 (Anema, 2009; Lucey et al., 1998). The peak in $\tan \delta$ occurs in any acid milk system, where gelation starts at pH values higher than 5.3 and continues to drop (Lucey, Tamehana, Singh, & Munro, 2000). The results of the current study showed that even though the gelation pH remained similar for the control and the enzyme-treated heated milk gels, no peak in $\tan \delta$ was observed in TG- and TrTYr-treated milk samples (Fig. 3b). We suggest that the

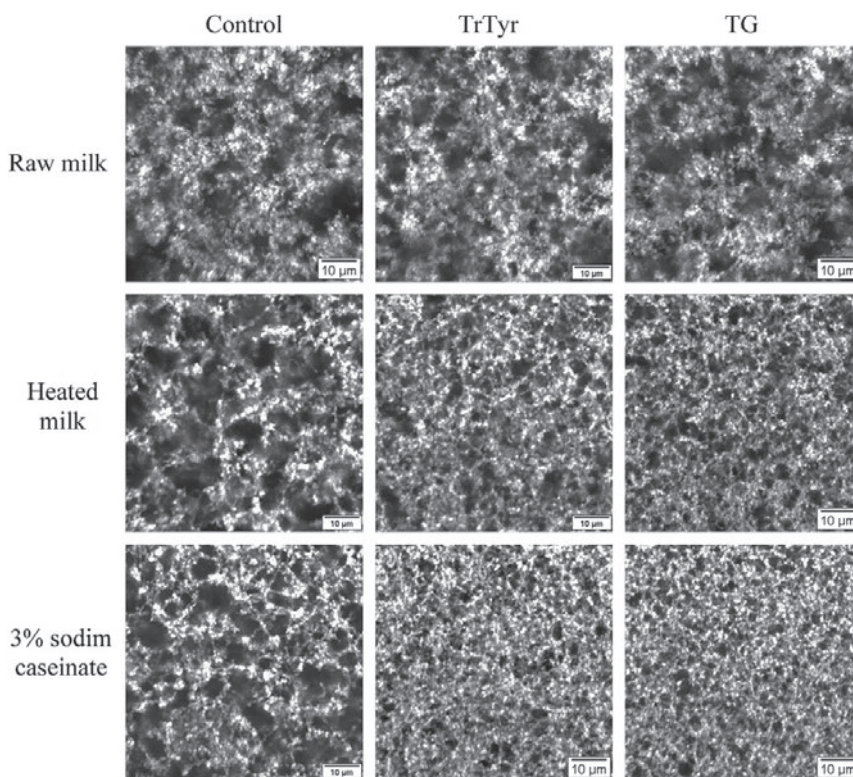


Fig. 5. CLSM images of acidified raw milk, heated milk and 3% sodium caseinate gels. Before acidification, samples were treated by TrTyr or TG for 1 h at 40 °C. Control samples were without any enzyme.

reason for the absence of a peak in $\tan \delta$ in the TG- and TrTyr-containing gels is that the cross-linking before acidification mainly occurs within the micelles resulting in increased micellar integrity upon solubilization of CCP. Formation of highly stable casein micelles upon intra-micellar cross-linking of caseins by TG has been reported (Huppertz & De Kruif, 2008; Smiddy, Martin, Kelly, de Kruif, & Huppertz, 2006). After cross-linking, CCP could be removed from the micelles without compromising the micellar integrity and the remaining structures were referred to as 'casein nano gel particles' (Huppertz & De Kruif, 2008). SEM images revealed considerable reduction in size of the particles due to TrTyr and TG in heated milk gels (Fig. 6). Reduction of particle size could also be an indication of conserved micellar integrity upon CCP solubilization and subsequent alteration in casein–casein interactions during pH drop.

In raw milk, TrTyr was the only enzyme which cross-linked caseins and caused an increase in the firmness of the acidified milk gel. Even though the extent of cross-linking was limited, a three-fold increase in the final G' (Table 1) and almost a two-fold increase in area under force versus distance curve (Fig. 4a) was obtained compared with the control raw milk gel. However, the control and TrTyr-treated raw milk gels were found to have only very slight differences in CLSM images (Fig. 5). The main effect of TrTyr in raw milk gel structure was identified in the morphology of the casein particles forming up the gel network as shown by SEM (Fig. 6a). TrTyr-treated raw milk gel was composed of considerably smaller casein particles compared with the TG-treated raw milk gel.

In heated milk, TrTyr did not show any effect on the final G' and firmness (Figs. 3b and 4b) even though it cross-linked the caseins to the same extent as TG. However, a lower final $\tan \delta$ was attained as compared with control. The difference in final $\tan \delta$ between the control and TrTyr gel indicates some change in the overall state of bonds in the network and viscoelastic character of the gel. Furthermore, CLSM image of the TrTyr-treated heated milk was similar to TG-treated heated milk, with small aggregates close to each other and smaller and more homogenous pores compared with control (Fig. 5). Three dimensional structure of the gel and curvature of the strands making the network affect directly the shear modulus (Lakemond & van Vliet, 2008a, 2008b). In that sense, the morphology of the cross-linked micelles resulting from TrTyr and TG treatments may differ from each other and thus affect the evolution of the storage modulus even though covalent cross-links exist in both systems roughly in the same extent. At this point, SEM microscopy was useful to visualize the assembly of the protein particles in the TrTyr- and TG-treated heated milk gels. SEM images revealed that TrTyr treatment did not improve the connectivity of the protein particles forming up the gel (Fig. 6c, d). Protein particles were close to each other like aggregates of individual particles. However, in TG-treated heated milk gel, the particles were fused into each other suggesting the presence of more efficient inter-particle interactions creating strong strands. Such a difference between the particle interactions would explain the difference between the final G' and the firmness (large deformation) values of the two gels. It is challenging to explain the reasons of this finding. Vasbinder and de Kruif (2003) claimed that after cooling heat-

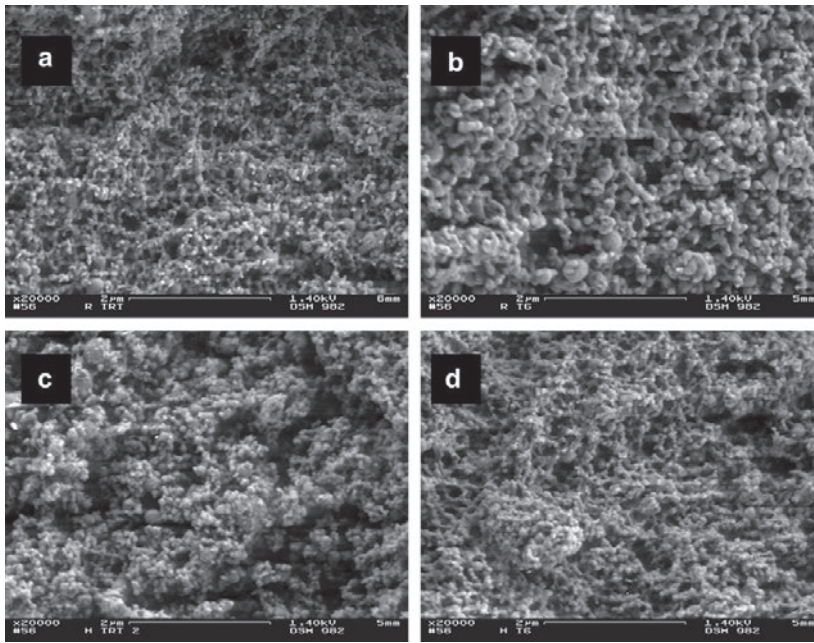


Fig. 6. SEM images of acidified milk gels: (a) raw milk + TrTyr; (b) raw milk + TG; (c) heated milk + TrTyr; (d) heated milk + TG.

treated milk, a number of thiol groups remain exposed and form disulphide linked protein structures during and after the gelation point. The authors studied gelation of heated milk with and without a thiol blocking agent and found that when the thiol groups were accessible, the gel firmness was significantly increased. Therefore, it is possible that TrTyr- and TG-pretreatment cause different structural properties in heated milk gels. Kato et al. (1986) have shown that dopaquinones, created by tyrosinase oxidation of tyrosine residues, can bind with sulfhydryl groups in proteins. It is probable that in TrTyr-treated heated milk, free thiol groups were at least to some extent intra-molecularly coupled to tyrosine residues either in β -lactoglobulin or κ -casein partially preventing the reformation of the disulphide bonds. As a result of this, the lack of additional disulphide bridges between the protein aggregates during gel formation might retard proper re-arrangements of the casein aggregates or affect the strength of the connectivity between the particles leading to low G' values. This phenomenon needs, however, further elucidation.

In sodium caseinate, the effect of TrTyr treatment on evolution of G' was rather similar to that of the TG treatment as was the extent of cross-linking. At neutral pH, in solution, sodium caseinate exists as self-assembled aggregates of different caseins (Ruis, Venema, & van der Linden, 2007) which gel upon decreasing pH towards their pI. Caseinate, with sub-micellar size aggregates of individual caseins which are not stabilized by CCP and lack of whey proteins, is a less complicated system compared with heated milk. It seems that TrTyr- and TG-treated casein micelles in milk behave differently during gelation, whereas in sodium caseinate, where native micelles are exchanged with sub-micellar size casein aggregates, two enzymes result in similar effects during gelling. In the case of sodium caseinate, the contribution of disulphide bridging on gel structure during and after the gelation point is negligible due to the lack of denatured whey proteins and relatively small concentration of κ -casein molecules compared with

other caseins. As a result, both TrTyr and TG treatments of sodium caseinate resulted in very comparable results in both firmness and microstructure.

5. Conclusions

Intriguingly, since milk protein cross-linking with *T. reesei* tyrosinase contrary to TG does not necessitate pre-heating of milk, this enzyme could be exploited in texture engineering of such milk products for which excessive pre-heating is undesirable. *T. reesei* tyrosinase was also found to be a superior enzyme compared with the commercially available *A. bisporus* tyrosinase.

It was shown that cross-linking of proteins (at the studied level) does not necessarily lead to increased gel firmness in milk gels. Cross-linking of micellar casein by different enzymes influences the morphology of the final protein particles forming the gel network and colloidal interactions between these particles, which determine the mechanical properties of the gel. A more thorough understanding on the mode of action of tyrosinase on micellar casein is needed. The type and location of the cross-links as well as the state of the free thiol groups upon tyrosinase action must be analyzed for more solid understanding on the role of tyrosinase in the highly complex structure of milk gels.

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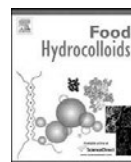
PUBLICATION IV

**Structural mechanisms leading
to improved water retention
in acid milk gels by use of
transglutaminase**

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Structural mechanisms leading to improved water retention in acid milk gels by use of transglutaminase

Dilek Ercili-Cura^{a,*}, Martina Lille^a, David Legland^{b,c}, Sébastien Gaucel^{b,c}, Kaisa Poutanen^a, Riitta Partanen^a, Raija Lantto^a

^a VTT Technical Research Centre of Finland, Tietotie 2, P.O. Box 1000, FI-02044 VTT, Finland

^b INRA, UMR782 Génie et Microbiologie des Procédés Alimentaires, 78850 Thiverval-Grignon, France

^c Agroparistech, UMR782 Génie et Microbiologie des Procédés Alimentaires, 78850 Thiverval-Grignon, France

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ABSTRACT

Water retention in transglutaminase (TG)-treated acid milk gels was studied and linked with the gel formation dynamics. Heat-treated skim milk with and without pre-treatment by TG was acidified at 20 °C, 30 °C and 40 °C at constant glucono- δ -lactone (GDL) level to obtain different acidification rates. Formation dynamics and structural properties of acid-induced gels were followed by rheological and near-infrared light backscattering measurements as well as microscopy. TG-treated gels showed decreased $\tan \delta$ values all through the acidification, which was pronounced around the gelation point. Backscattered light intensity was lowered in TG-treated gels compared to the controls indicating that TG-treated gels were comprised of smaller aggregates. Water holding capacity (WHC) was measured by using centrifugation at selected pH points (pH 5.2, 5.0, 4.8 and 4.6) during acidification. Both acidification temperature and TG treatment had significant effects on the water retention properties of the gels. Spontaneous syneresis observed at high acidification temperatures (≥ 30 °C) was prevented upon TG-treatment. WHC of TG-treated gels was significantly higher compared to the control gels at all pH points. TG-treated milk gels showed a homogeneous network formed of smaller aggregate and pore sizes at the gelation point and did not show any large-scale re-organisation thereafter. Transglutaminase is likely to act as a fixative of the protein network at an early stage of gelation and thereby limiting network rearrangements that take place in acid milk gels formed at high acidification temperatures leading to contraction and subsequent wheying off.

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1. Introduction

Structure formation during acid-induced gelation of heated milk has been extensively characterized and involves the following phenomena: 1. Gradual loss of electrostatic repulsion between the proteins as the pH approaches the isoelectric point of whey proteins, 2. Solubilization of colloidal calcium phosphate (CCP) from casein micelles upon pH reduction and simultaneous dissociation of micellar caseins (more significant at temperatures < 20 °C) (Dalgleish & Law, 1988; Gastaldi, Lagaude, & De La Fuente, 1996), 3. Aggregation of casein micelles, serum casein and whey proteins, which eventually form a non-rigid particle gel network, and finally, 4) re-organization of the aggregated protein clusters/strands towards equilibrium (Horne, 1999; Lucey & Singh, 1998; Phadungath,

2005). A structure as energetic as a gel can only be retained as re-organization becomes kinetically impeded.

Water in milk gels is physically trapped within the gel network meaning that the tendency for whey separation is primarily linked to dynamics of the casein network rather than mobility of the water molecules (van Vliet & Walstra, 1994). Walstra, van Dijk, and Geurts (1985) stated that the proneness of the casein network to large-scale rearrangements during and after the gel formation is the most important factor determining the water retention in such gels. The higher rearrangement potential of a casein particle network can be inferred for example from a high loss tangent ($\tan \delta$) value which indicates that relatively more protein–protein bonds relax and/or yield-reform (van Vliet, van Dijk, Zoon, & Walstra, 1991). The formation of a local maximum in $\tan \delta$ ($\tan \delta_{\max}$) at pH values around the maximum CCP solubilisation (\sim pH 5.2) (Dalgleish & Law, 1988) can be regarded as typical for heat-treated acid milk gels when gelation pH is above 5.2–5.0 (depending on the acidification temperature). Removal of the residual CCP and casein

* Corresponding author. Tel.: +358 40 015 7616; fax: +358 20 722 7071.
E-mail address: dilek.ercili@vtt.fi (D. Ercili-Cura).

dissociation cause loosening of the intra-micellar interactions while the micelle integrity is still preserved. The weakening of the intra-micellar structure leads to increasing $\tan \delta$ values which then start to decrease due to the enhanced electrostatic interactions creating a local maximum in $\tan \delta$ (Anema, 2009; Horne, 2003; Lakemond & van Vliet, 2008a; Lucey, 2002; Lucey & Singh, 1998; Lucey, Tamehana, Singh, & Munro, 1998). This behaviour is associated with high susceptibility of such gels (heated milk gels acidified at high temperatures) to large scale rearrangements at the initial stages of gel formation leading to spontaneous whey separation (Lucey & Singh, 1998; van Vliet & Walstra, 1994).

Structural properties of acid-milk gels can be modified by various factors such as pre-heat treatment temperature and duration, acidification temperature and rate or use of protein cross-linking enzymes. Modification of the protein matrix, which is mainly formed of weak physical interactions, directly by introducing strong covalent bonds within or between protein particles is one method to improve structural properties of skim milk gels. Use of transglutaminase (TG), a transferase which creates (γ -glutamyl)-lysine isopeptide linkages (Folk & Finlayson, 1977; Griffin, Casadio, & Bergamini, 2002), for that purpose has been extensively studied and reviewed (Buchert et al., 2010; Jaros, Partschfeld, Henle, & Rohm, 2006; Ozrenk, 2006). We have previously shown the effects of TG on texture and microstructure of acid-induced skim milk gels with the emphasis on the effect of pre-heat treatment (Ercili Cura et al., 2010). Clear indications on how cross-linking affects the rearrangement potential of the initial gel network was inferred through rheological measurements, i.e. loss tangent. However, the mechanism of how the covalent bonds introduced before or during the gel formation affect the aggregation dynamics of the micelles and the water retention properties of the subsequent gels was not studied. It has been stated that TG induces intermolecular cross-linking of the micellar caseins (Huppertz & De Kruif, 2008; Huppertz, Smiddy, & De Kruif, 2007; Schorsch, Carrie, & Norton, 2000). As a result, casein micelles are stabilized against dissociation (Huppertz & De Kruif, 2008; Moon, Hong, Huppertz, Fox, & Kelly, 2009; Mounsey, O'Kennedy, & Kelly, 2005) or disintegration due to removal of CCP from the micelles during acidification (Schorsch et al., 2000). As a consequence of modified casein micelle structure and micelle–micelle interactions, TG-treated milk gels are composed of altered gel particles organized in a lower mesh sized protein network and have increased mechanical strength (Ercili Cura et al., 2010; Schorsch et al., 2000).

In this study, the structure formation and water retention in heat-treated skim milk gels were elucidated by modulating the balance of contributing interactions. By combining the structural properties of the gels created by altered physical and chemical interactions to their ability to retain water, an attempt to identify particle and network properties that lead to better entrapment of water in acid-milk gels was made.

2. Materials and methods

2.1. Preparation of reconstituted skim milk

Low-heat skim milk powder (SMP) (34.5% protein, 53% lactose, 8% ash, 3.8% moisture, and 0.5% fat) was obtained from Valio Ltd. (Helsinki, Finland). Reconstituted skim milk was prepared by dispersing 9.57% (w/w) SMP in ultra-pure water by constant stirring with a magnetic rod at 50 °C for 1 h. It was tempered to room temperature (22–24 °C) with constant stirring in 30 min. Sodium azide (0.01% (w/w)) was added to prevent microbial growth. The reconstituted milk had a protein concentration of 3.3% (w/w). Prepared skim milk was heat-treated at 80 °C for 30 min. The heat treatment was performed in kimax tubes (inner diameter 13 mm),

placed in a water bath at 82 °C. At the end of 30 min (+2 min for the centre of the tube to reach 80 °C), the tubes were directly immersed in ice and the sample was cooled down to refrigeration temperature in 7–10 min. Heat-treated reconstituted milk was stored in a refrigerator overnight before use.

2.2. Enzyme treatment and preparation of acidified gels

Transglutaminase (Activa[®] MP, Ajinomoto) was supplied by Ajinomoto Foods Europe SAS (France). The activity of the enzyme was determined according to Folk (1970) and was 2500 nkat g⁻¹ of powder. Heat-treated reconstituted skim milk was tempered to 40 °C before addition of the TG. TG dosage was 100 nkat g⁻¹ of substrate protein in all cases. All samples (with and without TG) were incubated for 1 h at 40 °C prior to the addition of GDL. Acidification was carried out at three different temperatures, 20 °C, 30 °C and 40 °C. GDL was added at a constant dosage of 1.3% (w/w) once the samples were tempered to the desired acidification temperatures. Acidification was followed until pH reached 4.6.

2.3. Small deformation oscillatory measurements

Small deformation oscillatory measurements were performed by using a stress-controlled rheometer (StressTech, Reologica Instruments AB, Lund, Sweden). Milk samples at 20 °C, 30 °C or 40 °C were placed into the concentric cylinder (CC 25) measuring system (pre-adjusted to desired temperature) 5 min after GDL addition. Silicon oil was applied to the sample surface to prevent evaporation. All measurements were carried out until the sample reached pH 4.6. Samples were oscillated at a constant frequency of 0.1 Hz. The strain was kept constant at 1% which was tested to be within the linear viscoelastic region by performing strain sweeps at the end of time sweep measurements for each gel sample. The final G' and the final $\tan \delta$ refer to the G' and $\tan \delta$ values attained at pH = 4.6. The gelation time and the gelation pH were considered as the time and pH at $\tan \delta = 1$. Each measurement was performed on three individual gels. The pH profiles were followed simultaneously with rheological measurements by using a pH meter with a data logger (pH 3310, WTW, Weilheim, Germany). The pH meter was calibrated for the studied temperature. Both the rheological measurement and the pH meter were started 10 min after GDL addition.

2.4. Near-infrared light backscattering

Gel formation was also followed by a multiple light scattering technique using a Turbiscan[®] Lab Expert (Formulation, France) optical analyzer. The detection head of the instrument is composed of a pulsed near infrared light source ($\lambda = 880$ nm) and two detectors. The backscattering (BS) detector receives the light scattered by the sample at 135°. The gel samples were prepared as explained above and poured into special glass tube for gelation inside the Turbiscan[®]. The sample holder was pre-adjusted to the gelation temperature i.e. 20 °C, 30 °C or 40 °C. The measurements started 10 min after the addition of the GDL. A scan was performed every 5 min (10 min for the acidification temperature of 20 °C) until pH 4.6 was reached. The results represent the $\Delta BS\%$ in the mid-point (20–20.1 mm) of the glass tube.

2.5. Large deformation tests (penetration)

The firmness of the milk gels was measured with a TA-HDI Texture Analyser (Stable Microsystems Ltd., Godalming, UK) equipped with a 5 kg load cell. For this test, after the addition of GDL and 5 min mixing, the milk samples were poured (20 g) into plastic

cups with \varnothing 3.5 cm and a height of 6 cm (gel height \sim 2.3 cm) and incubated at 20 °C, 30 °C or 40 °C until pH reached 4.6. Before the test, gels were left at room temperature for \sim 10 min. Gel samples were deformed by penetrating a hemispherical plastic probe (\varnothing 1.27 cm) at a constant speed of 0.5 mm s⁻¹ to a distance of 70% of the gel height. The distance and force at rupture point were recorded on four individual gels for each sample.

2.6. Spontaneous syneresis

Spontaneous syneresis was defined as the ratio of the amount of water collected from the top of the gel at the end of incubation period (pH = 4.6) to the initial weight of the gel, expressed in percentage. The gels were formed in tightly sealed cups. The same cups defined in large deformation tests were used. Each cup contained 20 ± 0.2 g of sample. The water released was carefully removed and weighed. Results represent the average of five samples.

2.7. Water holding capacity (WHC)

For WHC analysis, the acid gels were formed in 50 mL centrifuge tubes. Each tube contained 20 ± 0.2 g of sample. WHC was analysed at four pH values during acidification; pH 5.2, 5.0, 4.8 and 4.6 by centrifuging the tubes at 3000 g for 15 min at 4 °C according to Imm, Lian, and Lee (2000). Before centrifugation, the tubes were taken from incubators (at 40 °C, 30 °C or 20 °C) and placed in ice-water to prevent further decrease of pH. The supernatant was drained carefully and weighed. WHC was defined as the ratio of the remaining pellet weight after centrifugation to the initial weight of the gel, expressed in percentage. Results are the average of five measurements.

2.8. Confocal Laser Scanning Microscopy

The microstructure of the acid gel samples were examined during gel formation with a confocal laser scanning microscope (CLSM, Bio-Rad, Hemel Hempstead Herts, UK) as described in Ercili Cura et al. (2010). The samples from the same gel were prepared in three different microscopy slides and each one was analysed at the selected pH values: pH 5.2, 5.0 and 4.6. Before analysis, samples were cooled to 4 °C by placing the slides in a refrigerator for 10 min. By cooling the samples, a further decrease in pH during microscopy was prevented. A 20 \times objective was used with zoom factor 5. The micrographs were acquired at 512 \times 512 pixel resolution (xy pixel = 0.25 μ m). Shown images represent overlays of 4 scans with 1 μ m steps at the same focal plane (depth = 4 μ m). The structures obtained were reproducible.

2.9. Analysis of microscopy images by grey level granulometry

Microscopical images were analysed using the grey level morphological granulometric analysis as described in Devaux,

Bouchet, Legland, Guillon, and Lahaye (2008). Grey level granulometry consists of image transformations based on a 'structuring element' with a given shape and size. *Morphological closing* is a transformation method which makes regions smaller than the structuring element disappear (Soille, 2003). By applying closings of increasing sizes to the original image, dark regions progressively disappear. $V(i)$ is built by measuring the sum of grey levels at each closing step i . The raw curve is normalised according to the initial and final sum of grey levels as follows:

$$g(i) = (V(i) - V(i + 1)) / (V(0) - V(\text{final})) \quad (1)$$

where $V(\text{final})$ is the sum of grey levels at the last closing step. Granulometric curves measure the proportion of grey levels that are modified between two successive closing steps. They can be compared to usual granulometric distributions except that they are calculated taking grey level variations into consideration. To facilitate their interpretation, curves were summarised by computing a grey level mean size, defined as the logarithmic mean of each curve (Legland, Devaux, Bouchet, Guillon, & Lahaye, in press).

$$\text{Size} = \exp\left(\sum_{i=1}^{\text{imax}} (g(i) \times \log(t_i)) / 100\right) \quad (2)$$

$g(i)$ is the percent of grey levels variations for the i th step, t_i is the size of the structuring element in μ m and imax is the number of closings.

2.10. Statistical analysis

Results were expressed as mean values and one-way ANOVA followed by an appropriate comparison of mean values test was used for statistical analysis (SPSS 14.0, SPSS Inc., Chicago, IL, USA) when necessary. Values were considered to be significantly different at $p < 0.05$.

3. Results

3.1. Oscillatory rheology and near-infrared light backscattering

Gel formation was followed with small deformation oscillatory measurements and the near-infrared light backscattering simultaneously for each sample. Acidification temperature affected the pH at gelation point ($\tan \delta = 1$). The gel point of the samples gelled without TG was identified at pH 5.54, pH 5.36 and pH 5.21 at 40 °C, 30 °C and 20 °C, respectively (Table 1). Higher acidification temperature resulted in the onset of gelation at higher pH values, which has also been previously shown by Banon and Hardy (1992) for the temperature range of 15 °C–42 °C, and by Haque, Richardson, and Morris (2001) for 40 °C–46 °C. TG treatment delayed the gelation time subsequently decreasing the gelation pH at all acidification temperatures (Table 1, Fig. 1).

Table 1

Parameters determined by small deformation oscillatory measurements and the near-infrared light backscattering during gel formation.

	Gel point (min)	pH at gel point	BS% at gel point	$\tan \delta_{\text{max}}$	pH at $\tan \delta_{\text{max}}$	Final $\tan \delta$	Final G' (Pa)	R (min ⁻¹)	Final BS%
40 °C									
Control	20	5.54	49.6	0.61	5.10	0.29 ^a	231 ^{ab}	0.23	57.9
TG	30	5.41	48.1	No peak	–	0.18 ^d	336 ^d	0.08	56.2
30 °C									
Control	73	5.36	47.8	0.48	5.10	0.26 ^b	204 ^a	0.10	58.3
TG	87	5.30	46.0	No peak	–	0.19 ^c	300 ^c	0.03	52.3
20 °C									
Control	240	5.21	43.7	No peak	–	0.23 ^c	207 ^a	0.03	52.7
TG	276	5.15	43.6	No peak	–	0.19 ^c	245 ^b	0.02	49.9

Values represented are means of three measurements except BS% which was replicated twice. Standard deviation was <1% for all measurements. Results with different superscript letters within the same column are significantly ($p < 0.05$) different from each other.

The effect of acidification temperature on final G' was not significant ($p < 0.05$), however, a significant ($p < 0.05$) difference between the $\tan \delta$ values at pH 4.6 was detected (Table 1). The values decreased from 0.29 to 0.23 from 40 °C to 20 °C. A peak was observed in $\tan \delta$ ($\tan \delta_{\max}$) at pH 5.1 when acidified at 40 °C and 30 °C, but not at 20 °C. The intensity of the $\tan \delta_{\max}$ at 40 °C was higher compared to that at 30 °C (Table 1). The more viscous character at 40 °C was also reflected to G' as a shoulder around pH 5.1. The increase in $\tan \delta_{\max}$ value and formation of a G' shoulder only at elevated (40 °C) acidification temperatures was in accordance with Anema (2009). When milk was TG treated, the final G' values increased significantly ($p < 0.05$) compared to the control gels at all temperatures (Table 1). However, a more substantial increase was expected which will be discussed later. In fact, the difference between final G' values of the control and the TG-treated gels at 20 °C was very small, but strain sweep conducted at the end of gel formation (at pH 4.6) showed that the control gel yielded before a strain amplitude of 0.1 while the TG-treated gel showed strain hardening behaviour and did not yield even after a strain of 1 (Fig. 2). The effect of TG treatment was also clear on final $\tan \delta$ values. At all acidification temperatures, the TG treated samples attained a final $\tan \delta$ of ~ 0.19 (Table 1) which was significantly lower compared to the controls. The other observation was the absence of a $\tan \delta_{\max}$ in TG-treated samples, which was also reported in our previous study (Ercili Cura et al., 2010).

In near-infrared light backscattering measurements, a local peak in BS% was observed at the gelation point for all samples. Castillo, Lucey, and Payne (2006) have also reported the local peak in light

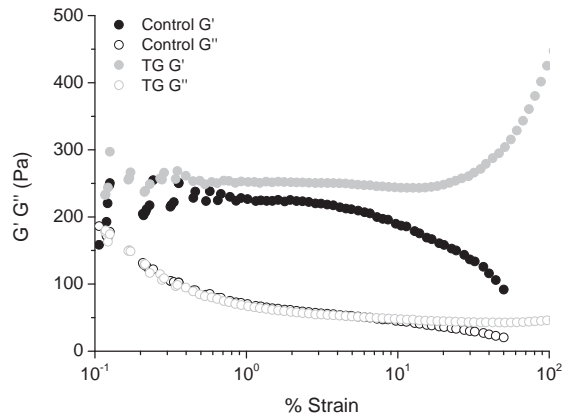


Fig. 2. Strain sweep of the final gels (at pH 4.6) acidified at 20 °C without (black symbols) or with (grey symbols) 100 nkat g⁻¹ TG.

backscatter ratio profiles of gels formed by the combined action of rennet and acid (mixed gels) and associated it with the start of the gel formation. BS% increased steeply after the gelation point and levelled in time (Fig. 1). At the acidification temperature of 40 °C, the control sample showed a second local peak in BS% profile, at around pH 5.1 which coincide with the $\tan \delta_{\max}$ (Fig. 1A). In TG treated

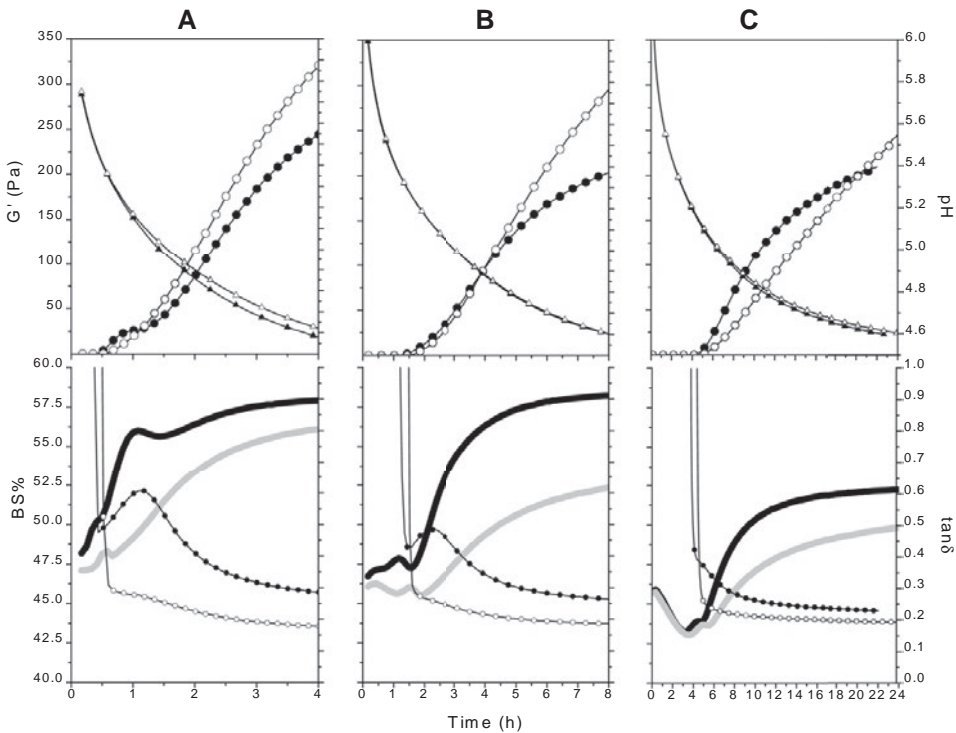


Fig. 1. Parameters determined by small deformation oscillatory measurements and near-infrared light backscattering during gel formation at acidification temperatures of 40 °C (A); 30 °C (B); and 20 °C (C). Storage modulus (G') (\bullet , \circ), and pH profile (\blacktriangle , \triangle) (upper row); $\tan \delta$ (\bullet , \circ) and backscattered light intensity (BS%) (black and grey lines) (lower row) were depicted for the control (solid symbols; black line); and TG-treated (open symbols; grey line) samples.

Table 2

Parameters (force at rupture point and distance to rupture point) obtained from large deformation measurements performed on the final gels (pH = 4.6) acidified at 40 °C, 30 °C, or 20 °C. Spontaneous syneresis levels are also shown for each gel.

	Force at rupture point (N)	Distance to rupture point (mm)	Spontaneous syneresis
40 °C			
Control	0.20 (0.01)a	5.74 (0.43)a	12.9 (2.3)
TG	1.38 (0.08) ^{b,c}	13.06 (0.46) ^{b,c}	0.0
30 °C			
Control	0.15 (0.00)a	4.99 (0.08)a	6.7 (0.4)
TG	1.10 (0.03) ^d	13.02 (0.18) ^c	0.0
20 °C			
Control	0.05 (0.00)b	3.73 (0.18)b	0.0
TG	0.34 (0.02) ^e	8.40 (0.45) ^d	0.0

Standard deviations are shown in brackets. Values with different letters within each column are significantly different ($P < 0.05$).

^a Sample did not rupture at the measurement conditions.

sample, only the peak corresponding to the gelation point was observed and the second peak was absent as was the $\tan \delta_{\max}$. At the acidification temperature of 30 °C, the initial BS% of the control sample started to increase until it made a local peak at the gel point (Fig. 1B). The TG-treated sample, however, initially showed decreasing BS% until the gel point. When the acidification was performed at 20 °C, the BS% of the control and the TG-treated samples decreased for the first 3 h until the gel point (Fig. 1C). The BS% values of the TG-treated gels were lower compared to the BS% of the control gels after the point of gelation up to the formation of the final gels at all acidification temperatures. One parameter derived from BS% data was the 'initial rate of network organization' (R). R was calculated from the slope of the linear part in BS% curves right after the gelation point. R was found to decrease with decreasing temperature/acidification rate, as expected (Table 1). When milk was TG-treated, R value was considerably decreased (2.8 fold at 40 °C, 3.7 fold at 30 °C, 1.8 fold at 20 °C) compared to the control gels.

3.2. Gel strength measured by penetration tests

According to the penetration tests, the control gel which was produced at 20 °C had a significantly lower firmness (force at rupture) and elasticity (distance to rupture) compared to the gels acidified at 40 °C and 30 °C, which were not significantly different (Table 2). There are inconsistencies in published results describing the effects of acidification temperature/rate on small and large

deformation rheological properties as pointed out by Anema (2008). Despite the differences in gel preparation conditions, our results were in agreement with the results reported by Haque et al. (2001) who have shown increased gel firmness (as determined by unidirectional compression) with increased acidification temperature (37 °C–46 °C). One point worth mentioning is the effect of spontaneous syneresis on penetration test results. The release of water from the control gels acidified at 30 °C and 40 °C might have resulted in a slight increase in the gel protein content, which could have had an effect on the force needed to break the gel.

The TG-treatment had a significant ($p < 0.05$) effect on the gel firmness and elasticity at all acidification temperatures as measured by large deformation tests (Table 2). The force at rupture values were seven folds higher for TG-treated samples compared to the related control gels for all temperatures. Likewise, the distance-to-rupture values were two-fold higher for TG-treated gels compared to the controls (Table 2).

3.3. Water retention

Spontaneous syneresis measurements revealed that 14% and 6% of the total weight of the gels acidified at 40 °C and 30 °C, respectively, were expelled as serum without any disturbance of the final gels. On the other hand, the gel acidified at 20 °C did not show any spontaneous syneresis (Table 2). At all temperatures, TG treatment completely prevented spontaneous syneresis.

WHC was studied during acidification at pH points 5.2, 5.0, 4.8 and 4.6 (Fig. 3). Both the acidification temperature and the TG treatment affected the WHC values at different pH points significantly ($p < 0.05$). WHC increased gradually with decreasing temperature at all pH points for the control gels. The same trend was observed for the TG-treated gels except at pH 5.0 where there was no significant ($p < 0.05$) difference between 30 °C and 40 °C-treated gels. The TG treatment significantly increased the WHC values at all pH points for all three acidification temperatures. While the control samples showed some variations between different pH points through acidification, the TG-treated samples did not show significant ($p < 0.05$) changes in WHC at different pH points.

3.4. Microstructure

The microstructure of the gels was analysed at three pH points: pH 5.2, 5.0 and 4.6. The micrographs obtained at acidification

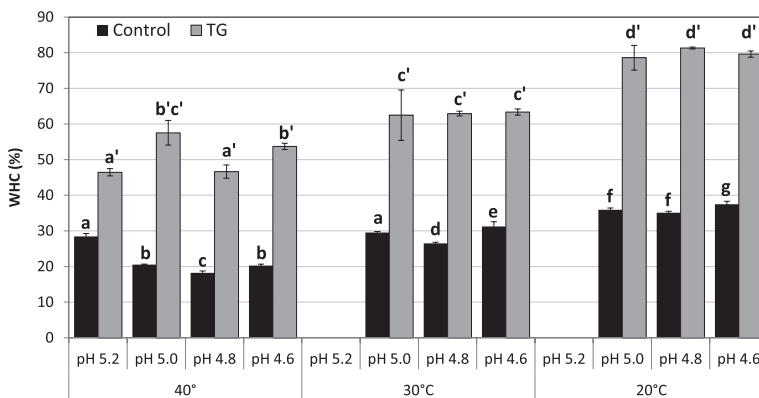


Fig. 3. Water holding capacity (WHC) of the control (black bars) and the TG-treated (grey bars) samples at different pH points during acidification at 40 °C, 30 °C, or 20 °C. Vertical bars represent the standard deviations. Statistical analysis was performed separately for the control and the TG-treated samples which was indicated by use of primed (') letters for TG-treated samples. Columns with different letters within each group are significantly different ($p < 0.05$).

temperatures of 40 °C and 20 °C both for the control and the TG-treated gels are shown in Fig. 4. The samples corresponding to acidification temperature of 30 °C were not shown as they were observed to fall in between the depicted images. Pore grey level mean sizes computed for each group of images are shown in Fig. 5.

At 40 °C, the micrograph of the control sample taken at pH 5.2 showed large protein aggregates with homogeneously-sized pores in between. The grey level mean size of pores was 3.3 µm. As the acidification proceeded, the gel network became coarser (at pH 5.0) with inhomogeneous clusters and pore sizes. The grey level mean pore size increased to 5.2 µm (Fig. 5A). The final gel at pH 4.6 showed that network rearrangements continued still resulting in an inhomogeneous structure with small and large pores (Figs. 4A and 5A). The TG-treated milk gel, however, attained a homogeneous and fine microstructure at all three pH points at 40 °C. More specifically, at pH 5.2, TG-treated sample revealed a small-mesh-sized fine structure and it did not evolve much at other pH values

until the final gel. Pore grey level mean size was around 3–3.5 microns. Only minor level coarsening seemed to take place (Figs. 4A and 5A).

At 20 °C, pH 5.2 was the point of gel formation for the control sample. In the micrograph, the initial forms of the protein aggregates were visible which are small-sized and show faint definition. As the acidification continued to pH 5.0, protein particles converted into a homogeneous network of particle clusters and did not show any further considerable changes at pH 4.6 (Fig. 4B). The grey level mean size of pores slightly increased from 3.2 µm at pH 5.2 to 3.8 µm at pH 4.6 (Fig. 5B). The TG-treated sample had a gelation pH of 5.15 when acidified at 20 °C, thus the micrograph taken at pH 5.2 shows nothing but slurry. At pH 5.0, the gel already attained a considerably fine structure which has the smallest-sized pores between the analysed samples (2.8 µm, see Fig. 5B). Also at this temperature, there was no dramatic difference between the micrographs taken at pH 5.0 and 4.6 for the TG-treated sample indicating the lack of any large scale rearrangements (Fig. 4B).

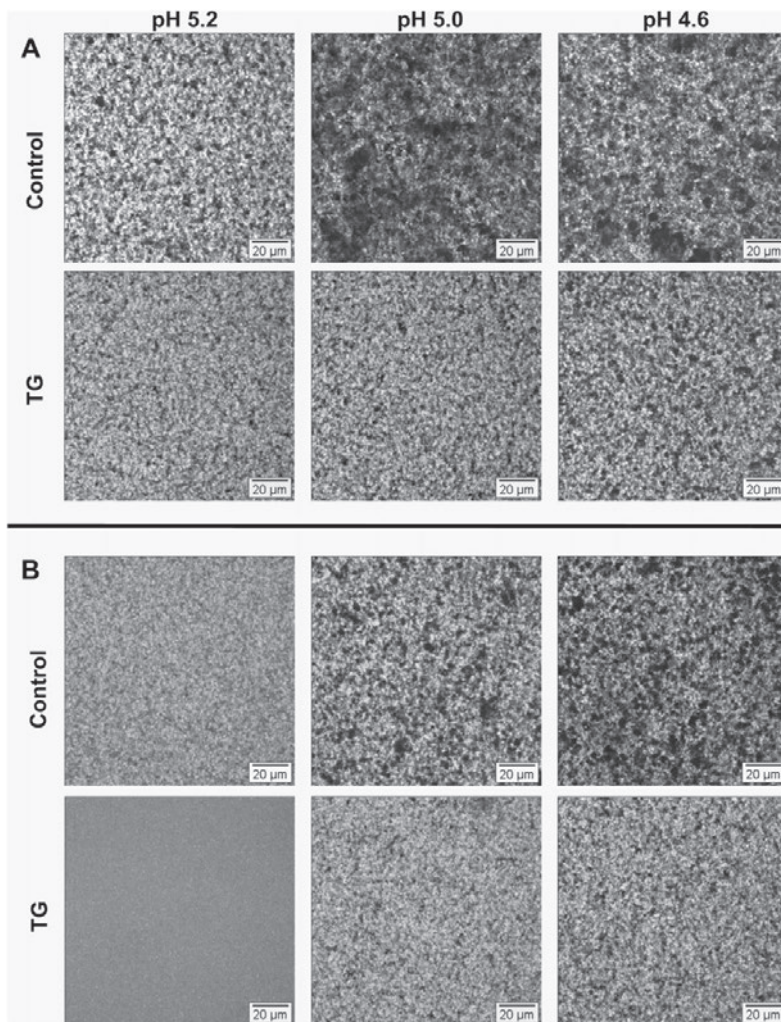


Fig. 4. Confocal Laser Scanning Microscope (CLSM) images of the control and the TG-treated acid milk gels at different pH points during acidification at 40 °C (A); and 20 °C (B).

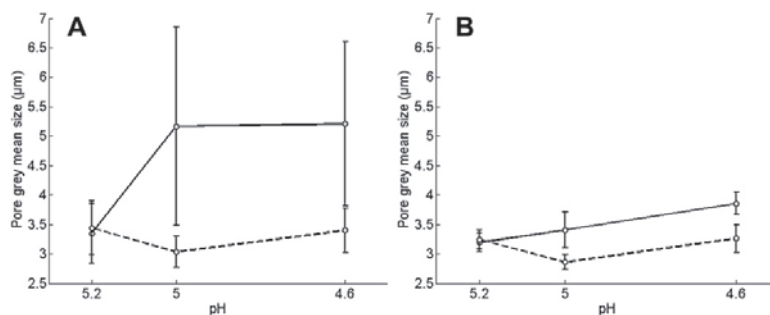


Fig. 5. Grey level mean sizes of pores obtained by image analysis on CLSM images of the control (solid lines) and the TG-treated (dashed lines) acid milk gels acidified at 40 °C (A) and 20 °C (B). Vertical bars represent standard deviation.

4. Discussion

The tendency of an acid-induced milk gel to exhibit syneresis is governed by the dynamic nature of the protein network. The structural and organizational properties of the fractal clusters and the extent of rearrangements at a constant protein concentration are very much dependent on the acidification temperature and the rate at which aggregation proceeds. In the present study, heated milk was acidified at different temperatures by using a constant GDL dosage in order to change the acidification rate and the contribution of the hydrophobic and entropic effects on the aggregation dynamics and on the final protein network. TG was used to create a distinct gel structure by incorporating covalent linkages to the protein matrix.

Proneness of a gel network to spontaneous syneresis is related to the extent of network rearrangements at the initial as well as later stages of gel formation leading to contraction of the protein network (Lucey & Singh, 1998; van Vliet & Walstra, 1994). Evidence for restricted rearrangements in TG-treated gels was obtained by following the gel formation process by rheological measurements, near-infrared light backscattering as well as microscopy. High $\tan \delta$ values at the initial stages of gel formation were previously associated with high relaxation or yielding potential exposing the strands and aggregates to fast rearrangements (van Vliet & Walstra, 1994). At acidification temperatures higher than 20 °C, the gelation started at pH values >5.2, thus there was considerable amount of residual CCP inside the casein micelles. Considering the high $\tan \delta$ values at the point of maximal CCP solubilisation (\sim pH 5.1, $\tan \delta_{\max}$), it can be inferred that the extensive rearrangements (with a decreasing rate in time) lead to release of water without any external force which was measured as spontaneous syneresis both at 40 °C and 30 °C. The $\tan \delta_{\max}$ was missing in the TG-treated samples indicating prevention of intra-micellar weakening and casein dissociation at the maximal CCP removal. This was also reflected to BS% results as loss of the second local peak observed at 40 °C. In the control gel which was acidified at 40 °C, a second local peak in BS% was observed which clearly coincided with the region of $\tan \delta_{\max}$ and the shoulder in G' . Castillo et al. (2006) have also detected the second local peak in light backscatter ratio of the mixed gels acidified at high temperature. Near-infrared light backscattering was found to be a valuable tool in detecting the gel point and obtain insight on the dynamics of the protein network at the initial stages of gelation. The rate of initial network formation was previously shown to be a relevant parameter in modelling the syneresis process as was measured by permeability in mixed gels (Castillo, Lucey, Wang, & Payne, 2006). Accordingly, a lower R value for initial rate of network organization in BS measurements would

correspond to lower levels of whey drainage in mixed gels. In the present study, TG treatment decreased the R values (2–4 folds) and prevented spontaneous syneresis in the gels acidified at high temperatures. The smaller R values and the lack of $\tan \delta_{\max}$ in TG-treated gels indicate that TG-treatment prevents the large-scale rearrangements already around the gelation point even at high acidification temperature/rate. The findings suggest altered reactivity of the micelles and strengthened inter-micellar interactions due to TG action. Furthermore, increased micellar integrity and constrained size due to extensive intra-micellar cross-linking might be causing altered aggregation kinetics.

An increase in BS% was previously associated with increasing aggregate size due to interactions between the micelles (Payne & Castillo, 2007). Even though the continuous release of CCP until $\tan \delta_{\max}$ could be affecting the refractive indices of both phases, the change in BS% after the gelation point has been correlated with micelle–micelle aggregate size (Castillo et al., 2006). Accordingly, the smaller values of BS% in the present study obtained for TG-treated gels as compared with control gels at each acidification temperature indicate a smaller size of the aggregates, which is well in accordance with our previous study, where scanning electron microscopy images of TG-treated and control gels revealed a considerable difference in size of the gel particles (Ercili Cura et al., 2010). In the present study, the micrographs of the TG-treated gels showed only minor changes from pH 5.2 to pH 4.6 revealing the formation of a low-mesh-sized, homogeneous microstructure immediately after gelation point which showed only minor changes in the pore size. By making use of near-infrared light backscattering and image analysis on the CLSM images, the aggregate and pore sizes of the TG-treated gel networks were shown to be considerably smaller compared to the control gels all through gel formation. High rate of rearrangements lead to inhomogeneous network due to increasing size of the building blocks of the fractal clusters and simultaneously occurring large pores (van Vliet, 2000). Larger pores reveal increased propensity of a gel to show syneresis. The effect of TG treatment on syneresis has been studied by e.g. Schorsch et al. (2000), who found that syneresis of a micellar casein gel acidified at 50 °C in the presence of TG was greatly reduced. Microstructural evaluation in both studies revealed that a reduction in syneresis correlated with an increase in the homogeneity of the gel network (decrease in size of clusters forming the network) and a decrease in pore size. The WHC of the TG-treated gels were superior to the controls at all three acidification temperatures and the values did not show significant change from the initial stages of the gelation (pH 5.0) to the final gel at pH 4.6. The ability of the gels to retain water under centrifugal force reflects their structural length scales due to hydrodynamic flow and

capillary pressure as suggested by Hermansson (2008) and thus, is not related to the molecular/micelle scale arrangements responsible for the increasing elasticity but directly related to the gel coarseness.

When milk was TG-treated, the final G' values were higher compared to the controls at all temperatures. However, the relative increase was less than in several other studies which found substantial increase in the elastic modulus upon TG-treatment (Ercili Cura et al., 2010; Færgemand & Qvist, 1997; Lorenzen, Mautner, & Schlimme, 1999; Lorenzen, Neve, Mautner, & Schlimme, 2002). The observed discrepancies in G' results are difficult to explain as the elastic modulus, besides the number and strength of the bonds, depend on the strand shape (curved or straight) as well as the strand thickness (Lakemond & van Vliet, 2008b). At high TG-dosages, extensive intra-micellar cross-linking as well as inter-micellar cross-links could alter the distribution of the structural elements. In fact, even though the difference between final G' values of the control and the TG-treated gels was small, large deformation tests showed a significant increase in firmness upon TG-treatment at all acidification temperatures. A similar observation was reported by Jaros, Pätzold, Schwarzenbolz, and Rohm (2006) who suggested that the TG-induced crosslinks could restrict the formation of other intermolecular interactions and thereby limit the increase in G' . The strain sweep test (Fig. 2), also showed that the TG-gel resisted much higher deformations than the control gel and that the TG gel exhibited strain hardening behaviour. Strain hardening in fractal colloidal gels originates from the intrinsic strength of particles forming the strands in a network. During strain sweep, the flexible strands are first extended and finally elongation of the particles due to increasing strain causes increasing G' (Gisler, Ball, & Weitz, 1999). Strain hardening has also been reported for TG-treated whey protein gels (Eissa & Khan, 2005). The strain hardening behaviour of the TG-treated acid gel in the present work could originate from the intra-micellar but also inter-micellar covalent crosslinks as strain hardening is typically observed with gels formed by covalent crosslinks.

Despite the vast understanding via the current models (adhesive hard sphere, percolating network, fractal) used to explain acid-milk gel formation, the kinetics of aggregation has not been fully explained yet (Mezzenga, Schurtenberger, Burbidge, & Michel, 2005). Aggregation kinetics of caseins as particles has been studied by various light scattering (Ruis, Venema, & van der Linden, 2007) and neutron scattering (van Heijkamp et al., 2010) techniques giving more insight on the understanding of particle–particle and cluster–cluster interactions on the way to formation of a gel network. A model, linking the intra-micellar changes, particle–particle interactions and rearrangements during network formation to rheological properties and water retention considering the acidification kinetics (rate, temperature) in such gels is yet to be developed (Mezzenga et al., 2005).

5. Conclusions

Gel formation dynamics and water retention of the acidified milk gels was highly dependent on both acidification temperature and TG treatment. TG treatment was shown to restrict rearrangements of the protein network during the gel formation process, which in gels without TG caused a further increase in pore size and subsequent water release at high acidification temperatures. The TG-treated milk gels showed a fine structured protein network with strong interactions and high WHC already at the initial stages of gel formation. Even though the fusion of particles and strengthening of the network continued, the microstructure of the protein network and the WHC stayed unchanged in the course of acidification.

Treatment of heated skim milk with high dosage of TG led to extensive intra-micellar cross-linking which was evidenced by loss of $\tan \delta_{\max}$ during acidification and the strain hardening behaviour observed for the TG-treated acid gels. Aggregation dynamics of the TG-treated casein micelles and the altered nature of inter-micellar interactions (chemical and physical) during acidification lead to distinct gel structures in TG-treated acid milk gels.

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Title	Structure modification of milk protein gels by enzymatic cross-linking
Author(s)	Dilek Ercili-Cura
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