Interfacial cross-linking of β-casein changes the structure of the adsorbed layer

Riitta Partanen\textsuperscript{a,}\textsuperscript{*}, Pirkko Forssell\textsuperscript{a}, Alan Mackie\textsuperscript{b} and Eva Blomberg\textsuperscript{c,d}

\textsuperscript{a}VTT Technical Research Centre of Finland, P.O.Box 1000, FI-02044 VTT, Finland

\textsuperscript{b}Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

\textsuperscript{c}KTH, School of Chemical Science & Engineering, Drottning Kristinas väg 51, SE-100 44 Stockholm, Sweden

\textsuperscript{d}YKI, Institute for Surface Chemistry, Drottning Kristinas väg 51, SE-100 44 Stockholm, Sweden

\textsuperscript{*}Corresponding author

Tel.: +358 40 825 5816

Fax: +358 20 722 7071

Email: riitta.partanen@vtt.fi
Abstract

The mechanism of transglutaminase-induced cross-linking of interfacial β-casein layer was investigated in tetradecane-buffer system. Monolayer studies were carried out in a Langmuir trough, where incubation with the enzyme mostly affected the compression of the film through adsorption of transglutaminase to the interface. Interfacial shear rheology was used to follow the kinetics of formation of a visco-elastic film upon cross-linking. Substrate concentration affected the rate of the interfacial cross-linking, when enzyme was dosed per protein concentration. This was most likely due to the saturated substrate layer at the interface in all cases. SDS-PAGE revealed that most of the β-casein at the interface was not cross-linked by intermolecular links, but rather, intramolecular links were formed. Finally, studies of adsorbed β-casein layers on polystyrene beads revealed that cross-linking reduced the thickness of the adsorption layer from 11-12 nm to 8-9 nm. These results suggest that it may be mainly intra-molecular cross-linking which modifies the physical interactions of β-caseins at the interface resulting in a higher layer density and thus, formation of a visco-elastic network.

Keywords: β-casein, interface, cross-linking, rheology, thickness
1. Introduction

Proteins contribute to food structure as three-dimensional networks in gels and solids but also as two-dimensional networks at interfaces of foams and emulsions. Interfacial properties of proteins have been a subject of considerable interest as very different films can be formed depending on the interactions and assembly of the protein molecules at the interface. Proteins with specific function on surfaces in nature provide unique properties, such as the extreme elasticity of hydrophobin films at air-water interface (Cox et al. 2009). In an attempt to improve the stability of food foams and emulsions, enzymatic means of tailoring interfacial properties of conventional food proteins have been sought for over a decade. While controlled hydrolysis can be used to improve foaming and emulsifying properties (Flanagan and Fitzgerald, 2002), increased interfacial elasticity and stability have been the aim using of cross-linking enzymes (Dickinson, 1997). The impact of enzymatic cross-linking on the properties of foams and emulsions suggest application potential (Ma et al., 2012; Macierzanka et al. 2011; Partanen et al., 2009; Dickinson et al. 1999), even if the effect depends strongly on conditions of preparation, either prior to or after cross-linking as well as on the extent of reaction (Dickinson et al. 1999, Flanagan&Fitzgerald, 2003). Improved foam stability has been found in case of active enzyme present at the time of foaming (Partanen et al., 2009). For emulsions, improved physical stability against Ostwald ripening (Dickinson et al. 1999) and improved stability against oxidation (Ma et al., 2012) has been reported. In addition, digestibility of protein has been shown to be altered by cross-linking (Macierzanka et al. 2012).

To gain more fundamental understanding of changes in the interfacial layer resulting from crosslinking, studies with model interfaces have been carried out. In particular, transglutaminase...
(TG, EC 2.3.2.13, \(\gamma\)-glutamyl-peptide, amine-\(\gamma\)-glutamyl transferase) has been used in these studies (Faergemand et al. 1997, 1998, 1999). A few other studies with tyrosinase (Ercili-Cura et al., 2012) as well as those with chemical cross-linkers have been reported (Romoscanu and Mezzenga, 2005; Tikekar et al. 2011). The results by Faergemand et al. (1999) show a 100-fold increase in surface shear viscosity of the interfacial films and in dilatational surface elasticity. Dependence of the build-up of surface visco-elasticity on enzyme dosage was also demonstrated, as application of high dose led to a steep increase, followed by a decrease in surface shear viscosity while application of lower doses were followed by a steady increase of the viscosity. While interfacial cross-linking of non-globular caseins has systematically been associated with increased visco-elasticity of the layer, results on interfacial cross-linking of globular proteins are more controversial (Faergemand et al. 1997; Sharma et al. 2002; Ercili-Cura et al. 2012), indicating the importance of motional freedom and availability of reactive sites in the substrate protein.

TG-induced reactions of milk proteins in bulk solution have been extensively characterized as a function of accessibility to the substrate as affected by heat, pH and reducing agents as reviewed by Jaros et al. (2006) and for side reactions such as deamidation under conditions of limited availability of the glutamine groups (Sharma et al., 2001). At high TG doses changes in electrophoretic mobility of monomers has been found, a property which has been linked with deamidation and intra-molecular cross-linking. The interfacial studies introduce further complications, as in these, the concentration of substrate is usually kept low in order to detect properties of the interfacial layer rather than effects from the bulk. Therefore, the incubation times are usually very long and/or the amount of the enzyme is above catalytic dosage meaning that it is not only affecting the system as a catalyst but potentially as a surface active protein.
In order to develop applications where stirred systems with lower enzyme dosages are more relevant, it is of importance to demonstrate the role of catalytic activity of the enzyme for the induced changes in the interfacial layer rather than contribution of the enzyme as a building block in the layer. Moreover, in order to develop multi-phase systems with interfaces promoting their stability, the structural changes taking place need to be assessed. Therefore the present study investigated the mechanism behind the increased visco-elasticity of the β-casein film at oil-water interface upon transglutaminase-induced cross-linking. The adsorption of the enzyme to the interface was studied in a Langmuir trough to understand more about the effect of the enzyme and its activity on β-casein monolayers. The effects of enzyme activity on protein oligomerization at the interface and on the dynamics of the reaction as affected by bulk protein concentration were studied by SDS-PAGE and interfacial shear rheology, respectively. Finally, the effect of transglutaminase on the thickness of β-casein adsorption layer on hydrophobic polystyrene beads was determined.

2. Materials and Methods

2.1. Materials

β-casein was purchased from Sigma (C6905, Sigma-Aldrich Finland Oy, Helsinki, Finland). Due to significant contribution of impurities to the interfacial tension during compression, tetradecane (Aldrich 87140, >99 % purity) was extracted 10 times with Milli Q-water prior to use. Hence, in the Langmuir trough, the contribution of purified tetradecane on interfacial tension at maximal compression was < 2mN/m, which was considered to be acceptable. The commercial Ca^{2+}-
independent TG product Activa WM (Ajinomoto Inc., Tokyo, Japan), was purchased from Vesantti Oy (Helsinki, Finland) and maltodextrin carrier was removed as described by (Lantto, Puolanne, Kalkkinen, Buchert & Autio, 2005). All other reagents used were of analytical grade. Throughout the study, beta-casein was studied in 10 mM phosphate buffer (pH 7.0), which was freshly filtered prior to the layer thickness measurements. Carboxyl latex beads (40 nm) were from Invitrogen (C37262, Life Technologies Europe BV, Bleiswijk, Netherlands).

2.2. Langmuir compression isotherms

An interfacial trough (KSV 5000, 362mm x 54 mm, KSV Nima, Finland) equipped with perforated barriers to allow flow of the upper bulk phase in compression was used to measure compression isotherms at 23.5 ± 1.0 °C. The cleanliness of the trough was first confirmed with water (π < 0.2mN/m), thereafter water was replaced by buffer solution and 38 µl of refrigerated β-casein solution (0.1 mg/ml) was evenly applied on the surface drop by drop with a Hamilton microsyringe. After equilibrating the surface for 20 min, tetradecane was carefully spread on the top outside the barrier with a glass pipette in contact with the trough wall until the Wilhelmy plate was submerged. The balance was zeroed, and the layer was equilibrated for 30 min before starting the compression at 1 mm/min. Transglutaminase was added either prior to compression (at an area per molecule of 8200 Å²/molecule for β-casein) or after partial compression (area per molecule of 5000 or 4000 Å²/molecule for β-casein). All calculations for molecular area were performed assuming negligible loss into the sub-phase during spreading the protein. In preliminary trials, spreading of cold protein solution led to a higher surface pressure in compression, which was taken as an indication of a higher retention of the protein on the surface. Enzyme was injected by two injections with a Hamilton syringe to the buffer sub-phase and
allowed to self-diffuse to the tetradecane-buffer interface. The amount of enzyme per interfacial area was equal to that used in rheological experiments with a protein concentration of 0.001%. The interface was incubated for 8 h with the enzyme prior to compression. The monolayer was first compressed to 1800 Å²/molecule for casein, expanded to 9400 Å²/molecule, where it was relaxed for 2 h, after which a second compression cycle was performed. In the case of o/w measurements, evaporation was not a problem, but in case of a/w studies, a control experiment with buffer alone was performed and found to account for an error of -0.2 mN/m per compression cycle due to evaporation of water.

2.3. Interfacial shear rheology

Interfacial shear measurements were carried out in an AR-G2 rheometer equipped with a DuNoüy ring (TA instruments, UK) with slight modification from Ercili-Cura et al. (2012). Visco-elastic properties of the layer at 25°C and β-casein concentrations of 10⁻¹ %, 10⁻³ % and 10⁻⁴ % were used in the studies. Exactly 45 g of the solutions was weighed in the measuring cup (Ø70 mm) which was placed on the bottom plate of the instrument. The ring was wetted in the solution and its position was adjusted to the interface. It is difficult to position the ring in a repeatable manner just by the eye. Consistent positioning in the measurements was ensured by weighing the liquid phase, and always using the same distance from the bottom plate. The proper position was determined in the preliminary experiments as the position of the ring which gave the highest value for elastic modulus. After adjusting the ring position, 10 ml of purified tetradecane was gently added on the top with glass pipette via the cup wall after which the time sweep was immediately started. Transglutaminase was added after 1 h in the sub-phase with a single
injection by Hamilton syringe in order to cause minimal disturbance to the formed interface. Transglutaminase was added at 50,000 nkat/g of β-casein in the case of constant enzyme / substrate ratio experiments. Experiments with constant enzyme / interfacial area ratio were also performed, in which the dose was the same for $10^{-3}$ % β-casein concentration, but 10-fold greater for $10^{-4}$ %, and 10-fold less for $10^{-2}$ % β-casein solutions. The linear visco-elastic region was confirmed for the final film by a frequency sweep and finally a strain sweep was used to evaluate the strain at which the interfacial layer ruptured. For time sweeps, a frequency of 0.005 Hz and 2% strain were used. Low frequency was used to improve sensitivity of the measurement for weak β-casein layers. Inactivated transglutaminase (o/n at 105°C), was used as a control.

2.4 Oligomerization of interfacial protein

Cross-linked β-casein interfaces were formed under the same conditions as the interfacial measurements. After 24 hour incubation at 23-25°C, the reaction was stopped by 15 min incubation with 0.45 ml of 1 M NH₄Cl. Then, 40 ml of the sub-phase was gently removed by a glass pipette with a narrow tip. The upper phase was gently moved into a concentration tube (Vivaspin 20, 3,000 MWCO PES). Centrifugation was performed at 6800 rpm for 4 h, after which excess tetradecane was removed from the top, and a 120 μl sample was taken from the aqueous phase above the membrane and mixed directly with 40 μl of sample buffer for SDS-PAGE. Tris-Glysine gel (12%, Lonza, Basel, Swtizerland) was used with pre-stained broad range standard, (Bio-Rad, Hercules, CA, USA) and β-casein and transglutaminase were injected on separate lanes to facilitate interpretation. Also, in addition to tetradecane buffer system, the reactions were performed on air-buffer system.
2.5. Layer thickness estimation

The thickness of the interfacial layer as affected by transglutaminase-induced reactions was studied by adsorption onto polystyrene beads with the method by Mackie et al. (1991). In order to maximize the resolution and prevent any competitive adsorption by bead dispersing agent, covalently stabilized beads (sulphonated) of 40 nm diameter were used. The size distribution of the beads was first measured in buffer solution by photon correlation spectroscopy (PCS) with Zetasizer ZS3600 (Malvern Instruments, UK), after which β-casein was added at different concentrations per calculated surface area of the beads. After overnight incubation without mixing at 23-25°C, the size distribution was measured again after which the enzyme was applied again followed by o/n incubation. The particle sizes were again measured and from the z-averaged cumulants, layer thickness was estimated by subtracting the radius of the original beads from the radius of the bead with adsorbed protein. Duplicate samples with triplicate measurements were made, of which average value and standard deviation was calculated.

3. Results and Discussion

3.1. π-A isotherms on Langmuir interfacial trough

The mean molecular area of β-casein was determined for air-buffer and for tetradecane-buffer interfaces in order to evaluate the monolayer properties of β-casein film. At the same mean molecular area (1800 Å²), a much higher surface pressure was observed for tetradecane-buffer interface (27.5 mN/m) compared with air-buffer interface (10.7 mN/m). Less difference between consecutive compressions was found for tetradecane-buffer system (Figure 1), where some
intermolecular interactions or orientation seem to remain after the first compression despite the relaxation step between the compressions. Compression of β-casein at air-buffer interface leads to a systematic irreversible decrease of surface pressure. The small difference in the starting levels of the second and third compressions of air-buffer interface is most likely due to evaporation of water from the trough during experiment. The differences between the air-buffer and tetradecane-buffer interfaces suggest that the protein molecules are more firmly attached to the oil-water interface.

Upon finishing each compression step, an immediate and steep decrease of the surface pressure was observed for both interfaces even if the barriers were only stopped and not expanded. This indicates most likely loss of protein due to desorption from the interface or major re-organization of the protein at the interface, such as expulsion of tails and loops of the protein as suggested by Mellema et al. (1998). At a 5 mm/min compression rate, a higher surface pressure was obtained in the end, indicating that the ability of the interface to organize during rapid compression was less, and the system was therefore further from the equilibrium (result not shown). Thus, despite the slow compression rate, equilibrium was not maintained during compression. Moderate hysteresis has been reported previously for π-A isotherms of β-casein (Dauphas et al. 2005). The equilibrium and non-equilibrium aspects of β-casein adsorption on tetradecane-water interface have been discussed by Maldonado-Valderrama et al. (2005). The authors have demonstrated the different interfacial conformations at oil-water interface and related the structural re-organization with interfacial coverage in equilibrium state. Given long enough time, protein interfaces re-organize not only in conformation, but depending on the bulk concentration they may also form multilayers, aggregates or gels on interfaces.
Transglutaminase was added in the sub-phase of equilibrated interfaces of β-casein in expanded and compressed states followed by 8 h incubation prior to the 1st compression (Figure 2A). It was hypothesized that some motional freedom in the interfacial layer is needed for cross-linking to take place. Incubation of the substrate protein layer with the enzyme at different degrees of compression resulted in difference in surface pressure levels. The treatment of the layers at 8200 Å²/molecule and 5000 Å²/molecule was followed by an increase in surface pressure, but the treatment at 4000 Å²/molecule had minor effect on the surface pressure of the layer. The subsequent compression of the layer treated at 4000 Å²/molecule is very similar to the 1st compression of the pure β-casein layer shown in Figure 1B. After relaxation of the cross-linked layers in expanded state for 2 h the differences had evened up (Figure 2B). Thereafter, surface pressure response to the 2nd compression of all the layers was nearly the same and considerably higher than the non-cross-linked β-casein layer.

Upon cross-linking, increasing intermolecular interactions could contribute to compressibility of the film in different ways: desorption of the protein from the interface could be hindered and also the film becoming more rigid, the proteins may be less able to re-organize at the interface upon compression. Both of these would be expected to lead to decreased compressibility, and thus, to higher surface pressure. In the experiments with TG, however, surface pressure already increased during the static incubation with TG, at surface pressures <15 mN/m. Yet another possibility is the formation of an elastic film, which may not be able to fully relax in the expanded state. However, build-up of elasticity indicating change in molecular interactions could not be observed in the shear rheology measurements during 8 h incubation with TG at corresponding
surface concentrations. These observations suggest the possibility that the enzyme can adsorb to the interface given enough time and space, up to a surface pressure of 15 mN/m. In our previous work with air-water interfaces, we have not observed any contribution of TG on the surface pressure of equilibrated β-lactoglobulin interfaces (Ercili Cura et al. 2012). In those experiments, surface pressure of the equilibrated substrate protein was 20 mN/m and thus above the critical limit indicated by the present study. In the present study, the adsorption of the enzyme to the interface would decrease the values of mean molecular area at the mixed interface, and thus shift the curves to the left (indicated by an arrow in the Figure 2B). For comparison, the compression of TG after 8h adsorption in plotted in Figure 2B. The position of the isotherm is theoretical, assuming 100 % efficiency in adsorption of TG. Still, this data reveals the presence of TG at the interface, given high enough concentration.

3.2. Interfacial shear rheology

The effect of cross-linking on the interactions of proteins and thus, visco-elasticity of the layer was studied by interfacial shear rheology. An equilibrated β-casein layer was too weak for elastic or viscous modulus to be measured, which is in accordance with its well-reported surfactant-like character (Faergemand et al. 1999). Upon addition of active transglutaminase however, both moduli started to build up simultaneously after a lag phase (Figure 3A and B). In all cases, build-up of both elastic (G’) and viscous (G”) moduli took place simultaneously but the elastic response dominated the behaviour. A maximum in surface shear viscosity, which was reported by Faergemand et al. (1999) for transglutaminase-induced cross-linking of β-casein, was not
observed for the viscous interfacial shear modulus in the present study. However, the films formed from lower concentrations had longer lag phases and were still developing at the end of the incubation time. The increase in viscous modulus had leveled off in the 0.0001 % system. Interestingly, not only the 0.001 % system with active enzyme but also the system with inactivated enzyme slowly developed a detectable viscous modulus.

Saturation of a β-casein layer at around 3 mg/m² is indicated by the work of Husband et al. (1997). In the present study, the most dilute concentration led to a theoretical surface concentration of 11 mg/m². Thus, the layer to be cross-linked could be saturated in all experiments, and therefore independent of the bulk concentration. As the enzyme was dosed based on the amount of total substrate protein in the system, the amount of enzyme was 100-fold for 0.01 % solution as compared with 0.0001 % solution. The higher rate of modulus increase was therefore to be expected. However, another experiment was performed, where enzyme was dosed per interfacial area (results not shown), i.e. equal amount of enzyme for all the samples, the rise in elastic modulus was fastest in the most dilute system. In order to act on the interfacial layer, the enzyme has to diffuse through the bulk phase. Possibly, the collisions with the substrate in the bulk and subsequent catalytic activity, which is not contributing to the interfacial but bulk rheology, reduce the rate of diffusion of the enzyme through the bulk solution.

The low frequency used in the time sweep was to minimize inertia in the measurement and thereby increase the sensitivity to change of a fluid layer into a visco-elastic film. However, as the oscillation frequency determines the time-scale of the molecular processes that are probed in the measurement, a frequency sweep (0.001-1 Hz) was performed after each time sweep (Figure
3C). Up to 0.1 Hz, there was slow, linear increase in G’. Towards higher frequencies, the increase in G’ became non-linear. Even in the high concentration case, where the increase of the moduli has leveled off in the time-sweep experiment, the film was still going through minor reorganizations. Similar mechanical spectra are also typical for 3D gel networks, which, in contrast to entangled networks show little dependency on frequency (Clark and Ross-Murphy, 1987). Also the strain sweep measurement (Figure 3D) confirmed the linear visco-elastic region for the time sweep, where build-up of visco-elasticity was followed. Most of the films collapsed between 20 and 40 % strain, but the lowest strain before collapse was 12 % for the 0.01 % system, and the highest stain before collapse was 60 % for the 0.0001 % system. Thus the higher elasticity of the film was generally associated with earlier breakage, but within the replicates deviation was high.

3.3. Extent of protein cross-linking by SDS-PAGE

The modulus could be increasing for two reasons. Firstly, if more protein becomes associated with the interfacial layer due to cross-linking, and secondly, if the interactions between the adsorbed proteins are changed. The covalent links forming between proteins are known to increase the hardness of transglutaminase-treated sodium caseinate gels (Mylärinen et al. 2007), and could be expected to work in a similar way in an interfacial layer. In order to evaluate the extent of intermolecular cross-links, the reactions were performed in the same vessel but without the oscillating probe, and the interfacial layer was separated from the bulk. The SDS-PAGE for tetradecane-buffer (lanes 7-8) and air-buffer (lanes 2-4) interfaces is shown in Figure 4. The
Intensities of bands between different cross-linked interfaces are not comparable due to the extraction and concentration treatments. The presence of transglutaminase enzyme (main band, lane 5) is observed at all interfaces. Only traces of native β-casein (lane 6) could be observed at the cross-linked interfaces. Instead, an intense band of protein with higher mobility than the native monomer was observed on all cross-linked samples (lanes 2-4, 7-8). This band is most likely due to formation of non-native, intra-linked monomers formed upon interfacial cross-linking. Such monomers could be formed via covalent links within single β-casein molecules, causing incomplete linearization of the protein by SDS and β-mercapto-ethanol. Similar behavior due to TG-induced cross-linking has been previously reported for ovalbumin and β-lactoglobulin by Giosafatto et al. (2012) and Ercili-Cura et al. (2012), respectively. The presence of non-native monomers, intra-linked monomers was suggested also in these studies. In size-exclusion chromatography, altered elution time of enzymatically cross-linked monomers has been demonstrated (Hellman et al. 2011). In the present study, cross-linked oligomers are found in addition to the intra-linked monomers. Due to multiple cross-linking sites for TG in β-casein, the oligomers appear as smear rather than distinct dimers, trimers and tetramers. As compared with the intensity of altered monomer band, the intensity of oligomers seems to be less. The presence of insoluble, high-molecular weight polymers remaining in the sample loading wells was not observed. Therefore, it is possible that rather than forming a continuously cross-linked network, transglutaminase is catalyzing formation of non-native monomers and small oligomers. Thus, the increased elasticity of the layer could be explained by physical interactions between these.

3.4. Thickness of the β-casein adsorption layer
In order to understand whether the change in rheology of the layer upon TG-induced crosslinking is due to increased amount of protein associated with the layer or due to the decreased repulsions within the existing layer, the effect of TG on the thickness of adsorbed β-casein layer was studied by PCS. Real oil-droplets are not suitable as an adsorption surface as they can become unstable, mainly through coalescence in the case of the present study. Thus carboxylated polystyrene beads were used as a stable hydrophobic adsorption media. The absolute thickness of the layer most likely differs to some extent from the layer at tetradecane-buffer interface due to the penetration of the protein into tetradecane. Small size of the beads was selected to keep them dispersed by Brownian motion, but also, to improve the resolution of the layer thickness measurement.

In Figure 5, the layer thickness values for β-casein adsorption layers before and after TG treatment are presented. The adsorption layer of β-casein was ca. 7 nm for 1 mg/m². At higher concentrations, 2-10 mg/m², the layer was saturated with 10-12 nm thickness. These values are in accordance with the study by Husband et al. (1997). In the present study, as TG was added to the bead dispersion with equilibrated adsorption layers of β-casein, a clear reduction in layer thickness was observed after overnight incubation. The dosage of the enzyme (1000 nkat/g or 10 000 nkat/g) did not affect the thickness of the layer, indicating that rather than acting as a building block in the layer, the catalytic activity of TG was of importance. At high dosage, however, particle size distribution revealed a minor fraction of aggregated material in addition to the individual beads with increased radius. This could be due to partial loss of repulsion or formation of covalent links between the beads due to TG activity. Reduction in the layer
thickness was small but repeatable. Further work would be needed to determine the precise origin of this densification.

In order to confirm that the reduction in layer thickness was not due to desorption of β-casein with time from the surface or due to the enzyme as a physically interacting macromolecule, control measurements for extended incubation without enzyme and with inactivated enzyme were performed at 5 mg/m² and 10 mg/m² concentrations (Table 1). Neither the inactivated enzyme nor the prolonged incubation time had an effect on layer thickness. These observations support the interpretation that the effect of TG on the adsorption layer is due to its catalytic action.
4. Conclusions

Given high enough concentration and “free” interface, TG was shown to adsorb on the tetradecane-buffer interface. However, with the low surface concentration of the β-caseins in the Langmuir trough studies, no elastic film could be formed by the addition of TG. At saturation concentrations of β-casein, TG-induced cross-linking was confirmed to lead to the development of visco-elasticity of the adsorbed layer. The build-up of visco-elasticity was explained by an increase in the density of the layer caused by increased interaction between β-caseins as observed in the adsorption layer thickness measurements. It is suggested that physical interactions between β-caseins also play a role in this process as little evidence for covalently linked three-dimensional network was found. Instead, β-casein oligomers and non-native monomers with altered mobility, most likely because of incomplete linearization due to the formation of intra-molecular cross-links by TG, were observed. Increased density of β-casein adsorption layer was indicated by the reduction in the thickness of the layer 11-12 nm to 8-9 nm due to cross-linking. In addition to the improved mechanical strength of the interfacial layer, the higher density may also protect the layer from desorption of β-caseins and reduce the contact between phases in an emulsion system. These may be important in consideration of mass transfer properties across the interface which affect not only physical but also oxidative stability of emulsions, and are crucial in development of emulsion-based encapsulation systems.
5. Acknowledgements

The work was carried out with financial support from the Academy of Finland in Project “Novel Protein-Based Emulsions by Engineering Interfacial Mass Transfer” (No. 133339). Heljä Heikkinen (VTT) is thanked for excellent technical assistance.

6. References


Table 1. Interfacial layer thickness for polystyrene beads incubated for two days with β-casein, or one day with β-casein and one with active or inactivated TG. Standard deviation (±) is given after the average value.

<table>
<thead>
<tr>
<th>Concentration (mg/m²)</th>
<th>Layer thickness (nm)</th>
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<tr>
<td></td>
<td>β-casein</td>
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<tr>
<td>5</td>
<td>11.6 ± 0.2</td>
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<td>10</td>
<td>11.2 ± 0.1</td>
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Figure captions

**Figure 1.** Surface pressure of β-casein in compression at 1 mm/min for A) air-buffer, and B) tetradecane-buffer interface: 1\textsuperscript{st} compression (solid line), 2\textsuperscript{nd} compression (dashed line) and 3\textsuperscript{rd} compression (dotted line). Average curves of the replicate measurements were plotted, where maximum surface pressure was 10.7 ± 0.2 mN/m for air-buffer interface, and 27.5 ± 0.5 mN/m for tetradecane-buffer interface.

**Figure 2.** Addition of transglutaminase in the sub-phase of equilibrated tetradecane-buffer interfaces of β-casein in expanded (8200 Å\(^2\)/molecule: dashed line), and compressed (5000 Å\(^2\)/molecule: dotted line; 4000 Å\(^2\)/molecule: dotted dashed line) states followed by 8 h incubation and subsequent compression. A) 1\textsuperscript{st} compression, the grey vertical line shows the starting point of compression after incubation. The curve below the discontinuity point is the compression prior to enzyme addition. Mean molecular area is calculated for β-casein. B) 2\textsuperscript{nd} compression, where the solid black line is for β-casein and solid gray line is for transglutaminase adsorbed from the sub-phase on equilibrated tetradecane-buffer interface with 8 h equilibration time prior to compression. The mean molecular area is calculated assuming that all the enzyme is adsorbing. The arrow shows the x-axis shift in the cross-linked curves in case of adsorption of transglutaminase.

**Figure 3.** Time sweeps of transglutaminase-treatment of β-casein films at 25 °C. Single measurement is shown and the final modulus value with standard deviation is given for each
sample after description. The time of TG addition is shown by arrow. Shear interfacial A) elastic and B) viscous modulus of β-casein at 0.01% (dotted line, $G' = 8.1 \pm 0.6 \text{ mN/m}$; $G'' = 2.0 \pm 0.2 \text{ mN/m}$), at 0.001% (dashed line, $G' = 5.9 \pm 0.6 \text{ mN/m}$; $G'' = 1.1 \pm 0.3 \text{ mN/m}$), at 0.0001% (solid line, $G' = 4.8 \pm 0.8 \text{ mN/m}$; $G'' = 0.4 \pm 0.1 \text{ mN/m}$), and at 0.001% with inactivated TG (dash dotted line, $G' = 0.2 \text{ mN/m}$; $G'' = 0.2 \text{ mN/m}$, single measurement). C) Frequency and D) strain sweeps for the cross-linked films. The grey vertical bars show the frequency and strain used in the other two sweeps.

**Figure 4.** SDS-PAGE gel interfacial layers of β-casein cross-linked with transglutaminase (TG) at 25°C overnight. Lanes: 1) molecular weight standard; 2) 0.0001% β-casein +TG (a/w); 3) 0.001% β-casein +TG (a/w); 4) 0.01% β-casein +TG (a/w); 5) TG control; 6) β-casein control; 7) 0.001% β-casein +TG (o/w) and 8) 0.01% β-casein +TG (o/w).

**Figure 5.** Thickness of β-casein adsorption layers on polystyrene beads. The symbols are for β-casein control (circles), for β-casein treated with 1000 nkat/g TG (squares) and β-casein treated with 10 000 nkat/g TG (triangles). Standard deviation is given in error bars.
Figure 1.

![Graph A](image1)

![Graph B](image2)
Figure 2.

A

B

Surface pressure (mN/m)

Mean molecular area ($\text{Å}^2$)

Surface pressure (mN/m)

Mean molecular area ($\text{Å}^2$)
Figure 3.

A

B

C

D
Figure 4.

Black and white image for printed version
Figure 5.
Highlights

- Cross-linking of β-casein with TG increases density of the adsorption layer.

- Interfacial cross-linking results mainly in intra-linking of proteins.

- In time and with free space available, TG adsorbs to o/w interface.
Interfacial cross-linking of β-casein changes the structure of the layer
Riitta Partanen\textsuperscript{a}, Pirkko Forssell\textsuperscript{a}, Alan Mackie\textsuperscript{b} and Eva Blomberg\textsuperscript{c,d}
\textsuperscript{a}VTT Technical Research Centre of Finland, \textsuperscript{b}Institute of Food Research, \textsuperscript{c}KTH, \textsuperscript{d}YKI,