

AN2002: Enzymatically cross-linked casein characterized by FFF-MALS

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Summary

Enzymatic cross-linking of caseins with microbial transglutaminase (mTGase) is a prominent example of protein polymerization and shows high potential for techno-functional applications in dairy processing. In many cases, the beneficial effects of the cross-linking were linked to the size of the large casein polymers generated by mTGase, and therefore high-resolution size separation techniques coupled to appropriate detectors are essential for a detailed molecular characterization.

This application note shows the capabilities of asymmetric flow field flow fractionation (AF4) in combination with differential refractive index measurement plus static and dynamic light scattering (AF4-MALS-DLS) for a gentle separation and investigation of caseins enzymatically cross-linked with mTGase. Using this approach, we have gained essential knowledge regarding the modus operandi of mTGase on size and conformation of caseins.

Introduction

Caseins (α S1 α S2, β , κ ; ~ 22 kg/mol), the main milk proteins, are responsible for the formation of structured gels in dairy processing. Enzymatic cross-linking of caseins with microbial transglutaminase (mTGase; EC 2.3.2.13; acyl transferase), which results in protein polymerization, has been extensively studied for enhancing the texture and physical properties of fermented dairy products such as stirred yogurt [1,2]. Size and molecular shape of casein polymers generated by mTGase have been reported to play a major role in the formation of optimal gel networks, but size determination by size separation techniques is very challenging because of the poor resolution and limited separation

range of size exclusion chromatography (SEC) and/or gel electrophoresis commonly applied for investigations of cross-linked caseins [3].

In this study, asymmetric-flow field-flow fractionation (AF4) was coupled to multi-angle light scattering (MALS), differential refractive index (dRI), and dynamic light scattering (DLS) detectors in order to gain a detailed characterization of molar mass, molecular size, conformation, and apparent density of caseins cross-linked with mTGase across a wider range of sizes than is accessible by SEC. This investigation elucidated, on a molecular level, the entire process of enzymatic cross-linking of caseins with mTGase.

Materials and Methods

Sample preparation

Acid casein powder (Sigma-Aldrich) was dispersed at 10 g/kg protein in 0.1 mol/L phosphate buffer (PBS) pH 6.8, and stirred overnight at room temperature; NaN_3 was added in a concentration of 0.3 g/kg to avoid microbial growth. The solutions were cross-linked with 3U mTGase (Activa MP, Ajinomoto Foods) per g casein at 40°C for 3h, and 24h; the reference (0h), instead, was not-mTGase treated according to the ref. [4].

AF4-LS analysis

An Agilent 1200 HPLC system equipped with an isocratic pump, auto-sampler, and degasser was used to control the flows to an Eclipse® DualTec® separation system; the eluent was the same used for sample preparation. Casein solutions were separated in an Eclipse long channel with a 490 μm spacer thickness and 10 kDa cut-off regenerated cellulose (RC) as ultra-filtration membrane. 10 μL of each sample solution was injected

by the auto-sampler into the channel. The channel flow was fixed at 0.5 mL/min for the entire AF4 run.

The sample solution was injected during the focusing/injection step with a focus flow rate of 3 mL/min for 4 min. A 2 min focusing/relaxation step was performed prior to the first elution step in which the cross-flow was kept at 2 mL/min for 5 min. Thereafter, the cross-flow was decreased linearly to reach 0.6 mL/min in 4 min, and then exponentially to reach 0.1 mL/min in 27 min. The cross-flow was kept constant at 0.1 mL/min for 15 min afterwards.

Elution of casein fraction was followed by an Agilent UV/Vis detector operating at wavelength of 280 nm, in combination with an 18 angle DAWN[®] MALS detector operating at a wavelength of 658 nm, an Optilab[®] differential refractive index (dRI) detector with a wavelength set at 658 nm, and online DynaPro[®] NanoStar[®] DLS detector connected via optical fiber to the DAWN.

Collection and processing of the light scattering data were performed using ASTRA[®] software. The scattering results were calculated using the Berry formalism and fitted with a first-degree exponential. A refractive index

increment (i.e. dn/dc) of 0.186 mL/g [5] was used for the determination of the molar masses.

Results and Discussion

As shown in Figure 1, the AF4-MALS method clearly reveals that caseins form relatively large aggregates in solution, with molar masses (MW) up to $\sim 10^6$ g/mol and radius of gyration (R_g) ranging from 10 nm to approximately 30 nm. Such aggregation is obviously a consequence of non-covalent interactions, i.e. hydrophobic and/or electrostatic interactions. However, when cross-linked, the MW of casein aggregates *increases* but the R_g distribution *decreases* to values that are smaller than 10 nm, which explains the shift of the maximum of the peak towards shorter elution times.

This counter-intuitive behavior indicates that mTGase leads to formation of structures that become less flexible as the cross-linking reaction proceeds, as confirmed by the conformation plots in Figure 2. Indeed, the scaling factor ν , which defines the conformation of the eluted molecules [3], decreases from 0.90 (elongated conformation) to 0.54 (more compact-coiled conformation) after 24h cross-linking time.

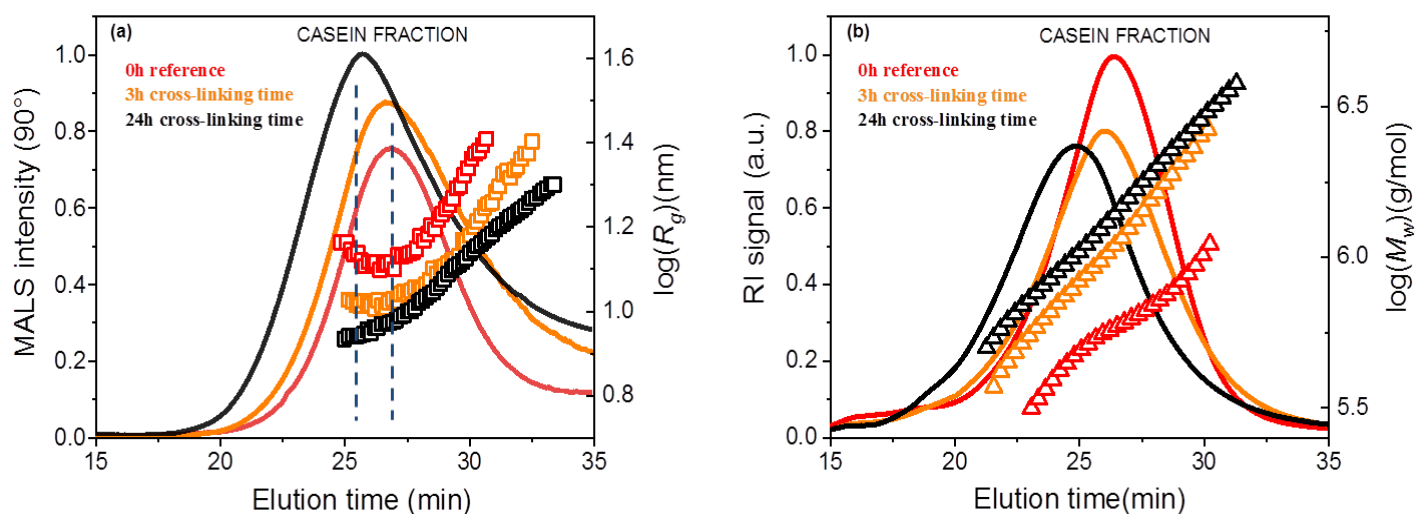


Figure 1. (a) MALS (90°scattering angle) signals and R_g distributions (\square), (b) RI signal (a.u.) and Mw distributions(Δ) vs elution time for casein fraction in 0h (red curves), 3h (orange curves), and 24h (black curves) samples.

Furthermore, the overall apparent density (d_{app}), calculated from the molar mass and R_g values determined by MALS, increases with cross-linking time (Fig.3). Interestingly, all samples shown in Fig.3 are characterized by a decrease of the apparent density with increasing MW, which indicates the formation of a loose network of caseins.

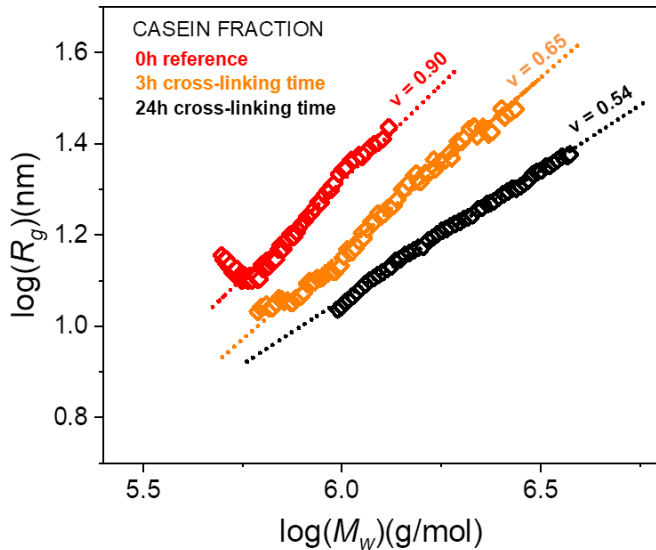


Figure 2. Conformation plots, i.e. log-log plot of R_g versus MW, for casein fraction in 0h (red curves), 3h (orange curves), and 24h (black curves) samples.

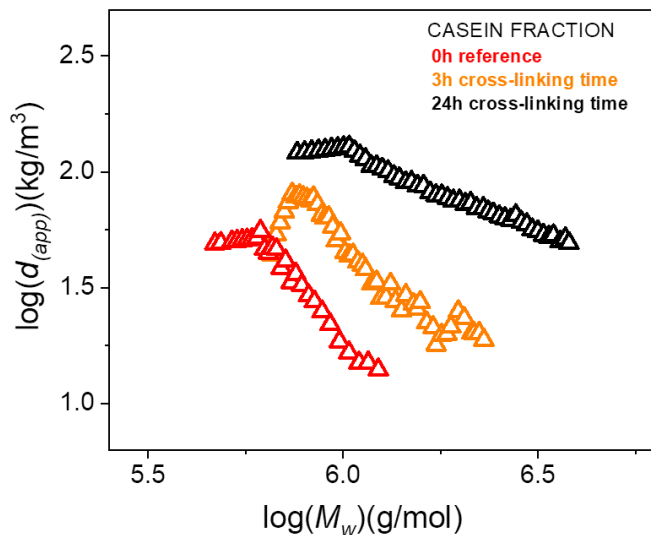


Figure 3. Log-log plots of apparent density (d_{app}) versus MW for casein fraction in 0h (red curves), 3h (orange curves), and 24h (black curves) samples.

As seen in Figure 4, we can combine the radius of gyration (from MALS detection) and hydrodynamic radius (R_h ,

from online DLS), i.e. R_g/R_h , which delivers insight into the shape of the cross-linked caseins [3]. It can be concluded that casein aggregates possess rather flexible structure before cross-linking (i.e. $R_g/R_h \sim 1.4$), but become nearly spherical after extensive cross-linking time ($R_g/R_h \sim 0.9$).

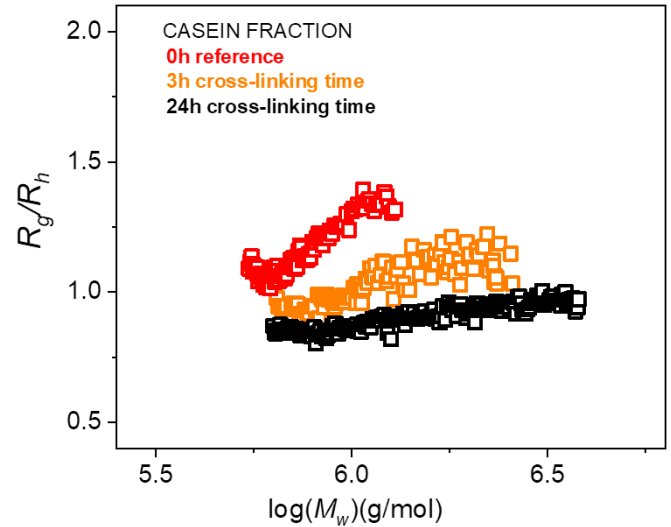


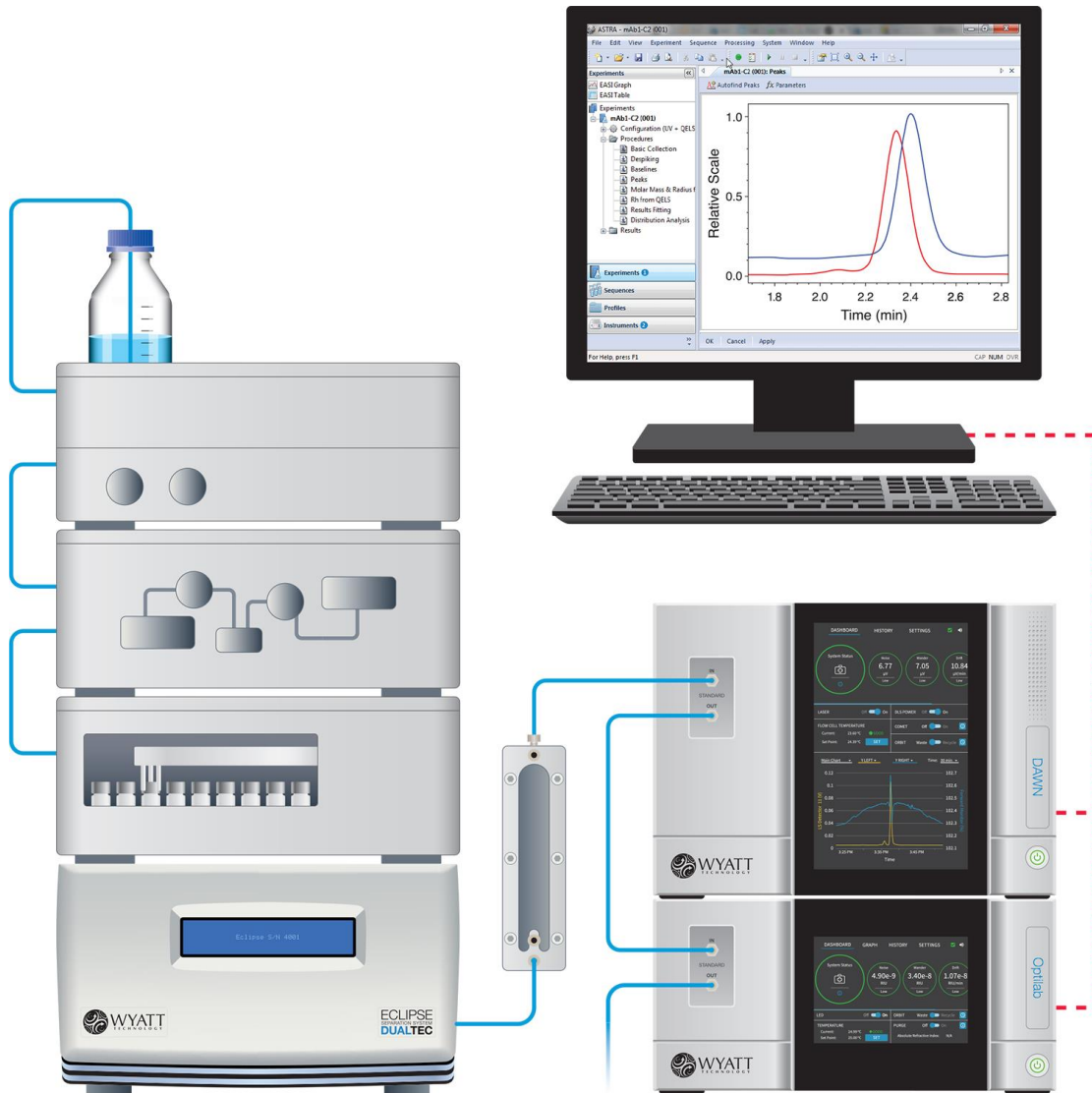
Figure 4. R_g/R_h versus $\log(MW)$ for casein fraction in 0h (red curves), 3h (orange curves), and 24h (black curves) samples.

Conclusions

This study demonstrates that AF4 in combination with MALS, dRI, and online DLS detections is an efficient method to investigate the cross-linking process of caseins with mTGase. The determination of molar mass, molecular size, shape and scaling parameters is highly relevant for understanding the relationship between molecular properties of cross-linked caseins and their impact on the techno-functional properties of dairy products.

References

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