Association of caseins with β-lactoglobulin influenced by temperature and calcium ions: A multi-parameter analysis

Hossein Mohammad-Beigi a,*, Wahyu Wijaya a, Mikkel Madsen a, Yuya Hayashi b, c, Ruifen Li d, Tijs Albert Maria Rovers a, Tanja Christine Jæger a, Alexander K. Bueß b, Anni Bygvå Ørnskov Hougaard d, Jacob J.K. Kirkensgaard d, h, Peter Westh b, Richard Ipsen a, Birte Svensson a, **

a Enzyme and Protein Chemistry, Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, Building 224, 2800, Kgs. Lyngby, Denmark
b Department of Molecular Biology and Genetics, Aarhus University, Universitetsparken 81, 8000, Aarhus C, Denmark
c Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Gustav Wiids Vej 14, 8000, Aarhus C, Denmark
d Department of Food Science, University of Copenhagen, Rolighedsvej 26, 1958, Frederiksberg C, Denmark
e Arla Foods Ingredients Group P/S, Sønderjyskvej 26, 6920, Vibelevsby, Denmark
f Protein Biophysics, Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, Building 227, 2800, Kgs. Lyngby, Denmark
g Niels Bohr Institute, Faculty of Science, University of Copenhagen, Blegdamsvej, 172100, Copenhagen, Denmark
h Interfacial Enzymology, Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, Building 224, 2800, Kgs. Lyngby, Denmark

* Corresponding author.
** Corresponding author.
E-mail addresses: hosmbe@dtu.dk (H. Mohammad-Beigi), bis@bio.dtu.dk (B. Svensson).

ABSTRACT

Aggregation of the major whey protein in bovine milk, β-lactoglobulin (β-Lg) is strongly influenced by association with caseins (CNs). Here, by using combined differential scanning fluorimetry and dynamic light scattering, the conformational stability and aggregation propensity of β-Lg and three types of CNs (α, β and κCNs) as well as their mixture have been systematically evaluated at different temperatures and Ca2+ concentrations in a multi-parametric approach. While β-Lg was affected significantly through denaturation and resulting aggregation by heat treatment with little dependency on Ca2+, αCN and κCN were influenced considerably by Ca2+. Through modifying the aggregation of β-Lg, CNs showed a different chaperone-like activity among the three types which were markedly dependent on the temperature and Ca2+ concentration. The presence of CNs resulted in smaller mixed aggregates compared to pure β-Lg aggregates, mainly through interaction of CNs with unfolded β-Lg and also by influencing the process of β-Lg unfolding. This was further confirmed by small angle X-ray scattering and isothermal titration calorimetry indicating that Ca2+ enhanced the interaction between β-Lg and CNs. Our experimental approach sheds light on molecular understanding of CN-β-Lg interactions and provides insight into how micro-structural assembly of milk proteins can be modulated to enable different functionalities in milk-based products.

1. Introduction

Interactions between caseins (CNs) and whey proteins during thermal processing of milk play a key role in the properties of dairy products. Milk and most beverages require heat treatment before sale or further processing (Etzel, 2004), which results in a number of physicochemical changes, including denaturation and aggregation of proteins (Durand, Gimel, & Nicolai, 2002). β-lactoglobulin (β-Lg) is the major whey protein (about 50%) in bovine milk and has a range of functionalities suitable for food and pharmaceutical applications, e.g. as an emulsifier (Li, Pan, Yang, Rao, & Chen, 2022) and wall material for encapsulation of bioactive compounds (Mekhloufi, Vilamosa, & Agnel, 2022; Zhang et al., 2022). During heat treatment, β-Lg undergoes conformational changes and aggregation, which are essential for some applications (e.g., gelation), but in certain cases, the aggregation is undesirable since it can destabilize the resulting food products. CNs, in particular, are naturally non-structured proteins that form macromolecular assemblies (denoted micelles) in an aqueous solution. It was noticed in the early 1960s
(Kenkare, Morr, & Gould, 1964) that CNs have chaperone-like behavior and can prevent aggregation of a number of proteins such as whey proteins, insulin, and ovalbumin and this versatility has been attributed to their highly flexible nature (Matsudomi, Kanda, Yoshika, & Moriwaki, 2004; Morgan, Treweek, Lindner, Price, & Carver, 2005; Yousefi et al., 2009).

Four types of CNs (αS1, αS2, β- and κCN) possess distinct primary structures and show different solubility in solutions containing calcium ions (Marchesseau et al., 2002). The inability of the CNs to form a stable tertiary structure stems from their high content of proline, which provides them with a flexible structure that renders them very susceptible to proteolysis (Fox, 2008). Additionally, post-translational modifications such as phosphorylation (in αS1, αS2, and βCN) and glycosylation (in κCN) lead to multiple proteoforms and heterogeneity for individual CNs (Fox, 2008). The combined features of CNs as lacking well-defined tertiary structure and having exposed hydrophobic residues confer a high surface activity (Li et al., 2022). Another interesting feature of CNs is the uneven distribution of the hydrophobic and polar residues (Fox & McSweeney, 2013), giving them an amphiphilic structure. Accordingly, different biophysical techniques are required to analyze the micellar characteristics and association of the mixtures of different forms of caseins, i.e. monomer, oligomer, and fibrils (Portnaya, Khafifin, & Danino, 2021; Wijaya et al., 2020). The high surface activity and amphiphilic structure provide CNs with good foaming and emulsifying properties and make them functional proteins for food applications. The CNs are present as large protein complexes (micelles) in milk. αS- and βCNs are assembled inside the micellar particles and stabilize the micelles through hydrophobic interactions as well as the interaction between their phosphoserine residues and calcium ions, while κCN is soluble in the presence of calcium ions and mostly situated on the micellar surface (Dalgleish & Corredig, 2012; Holt, Carver, Ecroyd, & Thorn, 2013). αS-CN and βCN lack cysteine, whereas αS2-CN and κCN contain half-cystine residues engaged in intermolecular disulfide bonds and association with β-Lg (Fox, 2005).

β-Lg is a small globular protein with a molecular mass of 18.4 kDa, which is present in different oligomerization states depending on the solution conditions. At neutral pH, β-Lg exists in a monomer-dimer equilibrium (Mercadante et al., 2012), which shifts toward the monomer with increasing ionic strength and temperature (Uhrínová et al., 2000), and undergoes irreversible changes at temperatures between 70 and 140 °C (Jametti, De Gregori, Vecchio, & Bonomi, 1996). The aggregation of β-Lg is also influenced by environmental conditions such as temperature, ionic strength and the presence of other proteins (Erabit, Flick, & Alvarez, 2013; Haque & Kinsella, 1988; Rodrigues et al., 2020; Sava, Van der Plancken, Cleays, & Hendrickx, 2005). It is not elucidated if β-Lg aggregates during the unfolding of the molecule or only at the end of the unfolding process. During unfolding, β-Lg exposes free thiols and hydrophobic groups, which are believed to induce the formation of links between molecules through intermolecular disulfide bridges (Petit, Herbig, Moreau, & Delaplace, 2011).

In the last few decades, extensive research focused on understanding the association of milk proteins and their unfolding and aggregation during the heating processes by using a variety of techniques. Previous studies have shown that CNs can diminish the aggregation of β-Lg as seen by a decrease in turbidity during heating (Guyomarc’h, Nono, Nicolai, & Durand, 2009; Matsudomi et al., 2004; Morgan et al., 2005; Yousefi et al., 2009), but the mechanism is not fully understood. A chaperone can prevent aggregation and aid in refolding of proteins, but dependent on the type of proteins, CNs behave differently and in some cases cannot assist in protein refolding and only prevent aggregation of unfolded proteins. The chaperone moity of CN micelles has been most frequently attributed to αCNs and βCNs (Morgan et al., 2005; Thorn et al., 2005; Yousefi et al., 2009). The hydrophilic domains of the CNs increase their solubility in aqueous media while their hydrophobic domains interact with partially unfolded β-Lg (Yousefi et al., 2009). Kehoe and Foegeding (2011) showed that βCN altered the heat-aggregation of β-Lg, as shown by a reduction in turbidity, through competing with whey proteins in the aggregation process, but differential scanning calorimetry (DSC) indicated that βCN did not alter the denaturation temperature of β-Lg (Kehoe & Foegeding, 2011). Furthermore, the same study showed that at 10 mM calcium chloride, chaperone activity of βCN was completely lost. Following this, Kehoe and Foegeding (2014) used transmission electron microscopy (TEM) to confirm that smaller and more regularly shaped aggregates formed when β-Lg was mixed with βCN, while the measured electrostatic properties of the final aggregates suggested that electrostatic stabilization was not the reason for the observed differences in aggregate size (Kehoe & Foegeding, 2014). Li et al. (Li, Pan, et al., 2022) used small angle X-ray scattering (SAXS) to show that the heat-induced denaturation and resulting aggregation of whey proteins is mainly a surface effect, leaving the overall protein shape unaffected, and that caseins remained largely unaffected. In addition, the interaction of β-Lg with CNs studied with graining incident SAXS (GISAXS) has shown that the core of the micelles becomes more compact as a result of preferential binding of β-Lg on the micellar surface (Steinhauer, Kulozik, & Gebhardt, 2014).

Although a wide range of different experimental techniques have been used to characterize the interaction of CNs and β-Lg, a single, recently developed microcapillary-based instrument platform, in which differential scanning fluorimetry is combined with dynamic light scattering (Prometheus Panta by Nanotemper), can address many of the central remaining questions on the protein mixtures’ thermoresistance and colloidal stability, solution homogeneity, and aggregation propensity. Here, we introduced the use of this instrument through performing thermal ramps from 20 to 95 °C to gain knowledge about the conformational state, thermal stability, and aggregate formation of milk proteins and their various mixtures. Simultaneous measurement of these characteristics allows the possibility of considering any interference from the interaction of the particles on the scattering and fluorescence signals. The thermodynamic and structural properties of the protein mixtures were determined by isothermal titration calorimetry (ITC) and SAXS.

## 2. Materials and methods

### 2.1. Preparation of protein solutions

β-Lg isoform A was purified in-house from raw milk (Kristiansen, Otto, Ipsen, & Quist, 1998) and found to be >95% pure as assessed by SDS-PAGE. αCN, βCN, and κCN from bovine milk (≥98, ≥70, and ≥70% protein, respectively) (Sigma Aldrich, St Louis, Missouri, USA) were used without further purification. Protein stock solutions (20 mg mL⁻¹) were prepared in 10 mM HEPES pH 7.0. β-Lg, αCN, βCN, and κCN concentrations were determined spectrophotometrically at 280 nm using molar extinction coefficients, ε, of 17,660, 25,900, 11,000 and 19, respectively.
0.35 M⁻¹ cm⁻¹, respectively.

Mixtures of the CNs and β-Lg were prepared by diluting and mixing protein solutions in 10 mM HEPES pH 7.0 containing different concentrations of CaCl₂. For the experiments at 85 °C, molar ratios of β-Lg/CN, β-Lg/CN, β-Lg/CN at 1:0.04, 1:0.2, 1:0.4, 1:0.8 were prepared at fixed β-Lg and CaCl₂ concentrations of 0.05 mM and 1 mM, respectively. Each mixture (2 mL) was transferred into a 4 mL glass vial for heat treatment (85 °C, 15 min) and subsequently immediately immersed in an ice-water bath for 10 min before performing further analysis.

2.2. Structure and scattering analysis of the proteins

The combined nano-differential scanning fluorimetry (nDSF), turbidity, and dynamic light scattering of the samples as a function of the protein and CaCl₂ concentration were carried out with a Prometheus Panta instrument (Nanotemper, Munich, Germany). The Prometheus Panta instrument provides simultaneous and multi-parametric assessments of structural integrity and colloidal stability of the proteins. Approximately 10 μL of each sample were loaded into microcapillaries and sealed with a specific sealing paste (Nanotemper, Munich, Germany) to prevent evaporation. The thermal unfolding profiles of proteins were measured by following the shift in the fluorescence emission spectra through the ratio of emission intensity at 330 and 350 nm (F350/F330) upon excitation at 280 nm caused by the change in the scattering intensity is increased by 10% (i.e. by a factor of 1.1). Third, the scattering intensity is calculated by a median fit to the initial data points (baseline). The samples were loaded into a rectangular quartz cuvette with a path length of 1 mm. The spectra of the three accumulated scans were averaged and corrected by subtracting the solvent/buffer spectra. The secondary structure of β-Lg/CN complexes was predicted using the instrument software, Spectra Manager™ (Jasco Int, Tokyo, Japan).

2.5. Composition of heated-treated protein solutions

Protein complex aggregates generated by heating of β-Lg/CN mixtures were fractionated and characterized by size exclusion chromatography (SEC) using a Hi-load Superdex 200 prep grade (pg) 16/60 column equilibrated at a 1 mL/min flow rate with 10 mM HEPES pH 7.0 and an AKTA explorer system (GE Healthcare, Chicago, Illinois, USA). Approximately 0.5 mL sample was loaded onto the column and the molecular weights of the proteins in the eluted fractions were calculated based on the retention volumes of the proteins and the calibration curve.

2.6. Determination of protein profiles and composition of protein complex aggregates

SEC fractions (see 2.5) were applied on Mini-PROTEAN® TGX™ Precast Gels (4–20%, BIO-RAD, USA) under denaturing conditions to analyze the composition of each peak. β-Lg/CN complex samples were applied (30 μL) to the well of a precast gel and electrophoresis was run using Mini-PROTEAN® System at 200 V for 30 min. With at least two changes, gels were stained for 1 h using Coomassie Blue and destained by soaking for at least 2 h in 10% CH₃COOH, 50% CH₃OH, and 40% H₂O. To evaluate the approximate sizes of proteins, molecular weight markers (Mark 12™ for native and SDS-PAGE were run along with the samples.

2.7. Small-angle X-ray scattering (SAXS)

Mixtures of β-Lg/CN, β-Lg/CN, β-Lg/CN were prepared by diluting and mixing protein solutions in 10 mM HEPES pH 7.0 containing 1 mM CaCl₂ to achieve mole-to-mole ratios of 1:1 for CNs and β-Lg at 0.1 mM β-Lg as fixed concentration. Each mixture (2 mL) was transferred into a 4 mL glass vial for heat treatment (85 °C, 15 min). Subsequently, the mixtures were immediately immersed in an ice-water bath for 10 min before SAXS analysis. SAXS was measured using a Nano-inXider instrument from Xenocs SAS (Grenoble, France) having a 40 W micro-focused Cu source (Rigaku-Denki, Co., Tokyo, Japan) producing X-rays with a wavelength λ = 0.154 nm. The Nano-inXider has a Pilatus 100 k pixel-detector from Dectris (Baden, Switzerland) measuring the scattering in a q-range from 0.005 to 0.3 Å⁻¹, where the scattering vector q = 4πsinθ/λ and 2θ is the scattering angle. 2D detector images were azimuthally averaged to produce 1D intensity curves as a function of q. Samples were measured in borosilicate capillary tubes with an inner diameter of 1.48 mm and the scattering from the buffer background was subtracted before analysis. Samples and backgrounds were measured for 45 min each. All background subtraction and initial data reduction were performed using the XSACT software package, including a logarithmic re-binning of the data.

All the SAXS data are presented in log I(q)- log q plots. Variation of scattering intensity I(q) within the sample can be calculated by:

\[ I(q) = Δρ^2 n_p V_p^2 P_p(q) S(q) \]

where Δρ², n₀, V_p, and P_p(q) are the contrast, number density, volume and form factor of the particle in question, respectively; q is the length of the scattering factor, and S(q) is the structure factor describing any interactions between particles. S(q) equals 1 in a dilute system, which we will assume here.

Subsequent data analysis and model fitting was done using the Sasview package (http://www.sasview.org/). Two fitting models were used, a model of ellipsoids was applied during the higher q ranges from 0.07 to 0.2 Å⁻¹ and a polydisperse spherical model to describe the larger
aggregates to fit the lower q range from 0.005 to 0.07 Å⁻¹.

The equations for ellipsoid model are as follows (Feigin, Svergun, & others, 1987):

\[ P(q, \alpha) = \frac{\text{scale}}{\psi} F(q, \alpha) + \text{background} \]

where:

\[ F(q, \alpha) = \nabla \rho V \left( 3 \left( \sin (qr) - q r \cos (qr) \right) \right) \left( qr \right)^3 \]

and:

\[ r = \left( R^2 \sin^2 \alpha + R_p^2 \cos^2 \alpha \right)^{\frac{1}{2}} \]

\( \alpha \) is the angle between the axis of the ellipsoid and q, and \( V = \frac{4}{3} \pi R_p R^2 \) is the volume of the ellipsoid, \( R_p \) is the polar radius along the rotational axis of the ellipsoid, \( R_e \) is the equatorial radius perpendicular to the rotational axis of the ellipsoid and \( \Delta \rho \) (contrast) is the scattering length density difference between the scatterer and the solvent.

The following polydisperse sphere model was applied to the scattering data in the q range from 0.005 to 0.07 Å⁻¹ (Yang, Tyler, Ahrné, & Kirkensgaard, 2021):

\[ P_{PS}(q) = \int P_{S}(q) D(R) V(R)^2 dR \]

where the monodisperse sphere form factor is given by.

\[ P_{S}(q) = \left( \frac{3 \left( \sin (qR) - qR \cos (qR) \right)}{qR} \right)^2 \]

2.8. Principle component analysis

Principal component analysis (PCA) was performed on each protein dataset (αCN, βCN, κCN and β-LG) to reduce dimensionality of the results and explore the relationship between protein/calcium concentrations and the responses quantified using Prometheus Panta (Tagg, I-Rad, I-Tur, E-Tur and P-Uns), Each dataset was log-transformed and scaled prior to PCA performed using the FactoMineR (ver. 2.4) (Le, Josse, & Husson, 2008) package in the R environment (ver. 4.2.0). Resulting biplots were visualized using the factoextra (ver. 1.0.7) package color-coded separately for the two treatment groups (protein concentration and calcium concentration). In each plot, results obtained at the same concentration of protein or calcium were clustered by a convex hull to show the concentration-dependent patterns. The alpha value (transparency) of the arrows (variables) refers to the percentage contribution of the variable.

3. Results and discussion

3.1. The effect of protein and calcium concentrations on CNs and β-LG properties

One of the most defining features of the CNs and whey proteins, which affect the final properties of the protein mixtures, is their
temperature-induced aggregation behavior in solution (Anema, 2021; Li et al., 2019). Some protein solutions become turbid upon heating above 90 °C. To determine the thermal stability, the proteins in HEPES buffer at pH 7 and at different concentrations (CNs 0.025–0.5 mM; β-Lg 0.05–0.25 mM) in the presence of CaCl$_2$ (0–10 mM) were heated from 20 to 95 °C at a scan rate of 1.5 °C/min. To evaluate the degree of refolding and reversibility of the assembly and/or aggregation of the micelles and β-Lg, the samples were subsequently cooled to 20 °C at the same scan rate (1.5 °C/min). An example of such experiments (for αCN) is shown in Fig. 1 and for the rest of the proteins in Figs. S1–S3. We extracted different information from this data, i.e. initial cumulant radius (I-Rad) and turbidity (I-Tur), turbidity at 95 °C (E-Tur), the temperature at which the average particle size began to increase, $T_{\text{size}}$, the onset temperature of heavy aggregation, $T_{\text{agg}}$, and ratio of emitted fluorescence intensities at 350 and 330 nm at 20 °C, which allows determining the thermal stability of the proteins (P-Uns) (Fig. 2 and Fig. S4). $T_{\text{size}}$ Fig. 2. Unfolding and size analysis of the proteins in the presence of CaCl$_2$ (0–10 mM). Cumulant radius of αCN (a), βCN (b), κCN (c) β-Lg (d). Relative turbidity of αCN (e), βCN (f), κCN (g) and β-Lg (h). Fluorescence ratio 350/330 nm of αCN (i), βCN (j), κCN (k) and β-Lg (l). The onset of increase in the cumulant size ($T_{\text{size}}$) of αCN (m), βCN (n), κCN (o) and β-Lg (p) and $T_{\text{agg}}$ of αCN (q), βCN (r), κCN (s) and β-Lg (t). The size of the aggregates in the presence of Ca$^{2+}$ was compared to that in the absence of Ca$^{2+}$. Accordingly, $T_{\text{size}} = 20$ °C and $T_{\text{agg}} = 20$ °C for the conditions in the presence of Ca$^{2+}$ in Fig. 2 m–t indicate that the proteins are already aggregated before the heating process. However, they show a distinct onset of further aggregation at higher temperatures. These parameters were measured by heating the proteins from 20 to 95 °C in the presence of CaCl$_2$ (0–10 mM). The concentration of CNs and β-Lg was 0.025–0.5 mM and 0.05–0.25 mM, respectively. The change in the protein structure and size of the proteins by heating and cooling processes are shown in Figs. S1–S3.
obtained by DLS exhibits the tendency of micelles to aggregate, which can correlate with thermal unfolding of proteins. The measured parameters varied greatly between the samples, suggesting that they represent a useful set of quantities to discriminate the different behavior between the investigated conditions.

In aqueous solutions, the CNs exist as monomer, micelle, or aggregated micelles dependent on the protein concentration, temperature, and Ca\textsuperscript{2+} concentration (Dauphas et al., 2005). To compare the overall patterns of protein properties between different protein and Ca\textsuperscript{2+} concentrations, a principle component analysis (PCA) was performed for the measured responses to changes in temperature (supplementary note and Figs. S5 and S6). The effect of Ca\textsuperscript{2+} on heat denaturation and aggregation of β-Lg and the CNs. Comparison of the dependency of unfolding and size of the proteins on Ca\textsuperscript{2+} at 20 °C. The dark and light colors in the color gradient indicate the highest and lowest Ca\textsuperscript{2+} effect on the property, respectively. Properties of αCN and β-Lg show the highest and lowest dependency on Ca\textsuperscript{2+} at 20 °C. Heat denaturation and aggregation of β-Lg (b), αCN (c), βCN (d), κCN (e) in the presence of Ca\textsuperscript{2+}. Ca\textsuperscript{2+} only binds the unfolded β-Lg, induces aggregation and shifts the equilibrium toward unfolded protein. Heating leads to the formation of highly stable β-Lg and αCN aggregates via disulphide covalent bond formation, which do not dissociate after cooling down. Ca\textsuperscript{2+} leads to the formation of polydisperse αCN and βCN micelles, but it has slight effect on the stability of the micelles and after cooling, the turbidity decreases. NS: Native state; US: Unfolded state. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Detailed analysis of the data showed that at 20 °C and in the absence of Ca\textsuperscript{2+}, the micelle or protein size was the same, regardless of the protein concentration (Fig. 2a–d). Although κCN micelles have slightly larger initial radius at 20 °C (17–19 nm) than βCN (9–12 nm) and αCN (5–7 nm) in the absence of Ca\textsuperscript{2+}, their size remains between 16 and 20 nm even at 10 mM Ca\textsuperscript{2+}. The initial size of βCN and αCN is strongly influenced by the protein/Ca\textsuperscript{2+} ratio, which is dependent on the stoichiometry and correlated with the presence of respectively 5 and 10 phosphoserine (PS) groups on βCN and αCN. The ratio of CaCl\textsubscript{2} to PS is listed in Table S1. Ca\textsuperscript{2+} at low concentration, but at a molar ratio of at least 4 to αCN and βCN could increase the size of the micelles at lower protein concentration (0.025 mM). However, with increasing CNs concentration more Ca\textsuperscript{2+} albeit, at a lower ratio to PS (around 1), was required to increase the size of protein aggregates. As αCN contains more PS than βCN, higher Ca\textsuperscript{2+} concentration is required before the size of αCN is not changing anymore.

This pattern of Ca\textsuperscript{2+}–dependence is represented in the PCA analysis, where the positive correlation between Ca\textsuperscript{2+} and I-Rad appears stronger at higher Ca\textsuperscript{2+} concentrations (2.5–10 mM) particularly in the case of βCN (Fig. S5). On the other hand, lower Ca\textsuperscript{2+} concentrations (0–2.5 mM) were associated with negative PC1 scores based on high Tagg and/or P-Uns, indicating that αCN and βCN are intrinsically tolerant to high temperatures in the absence of Ca\textsuperscript{2+} while κCN did not show such a clear trend. The increase in the size of αCN and βCN by adding Ca\textsuperscript{2+} can stem from the combined effect of the Ca\textsuperscript{2+} bridging PS groups of proteins and shielding negative charges, together decreasing electrostatic repulsion (McSweeney & Fox, 2013). It should be noted that the polydispersity index (PDI) is protein dependent, and ranked in the following order αCN > βCN > κCN > β-Lg. The micelles are more polydisperse at higher protein concentrations (Fig. S7). In line with this observation, in PCA, E-Tur was in most cases positively correlated with an increase in the protein concentration at all Ca\textsuperscript{2+} concentrations (see Fig. S6, with a particular focus on the PC2).

Turbidity measurements provide an indication of the presence of large (100s of nm to micron-sized). The sample turbidity was quantified...
by measuring the intensity of back-reflected, i.e. not scattered light, which depends on the concentration and size of aggregates. The sample turbidity increases upon heavy aggregation and/or significant increase in the size of the particles. The turbidity data at 20 °C (I-Tur) show the dependency of the aggregation of CNs and β-Lg on Ca2+ in the following order: αCN > βCN > αCN > β-Lg (Fig. 2 e-h). A comparison of the turbidity and DLS data can provide an overview on the population of small and big particles. While DLS can be biased by the presence of a small number of big particles, and provide the false impression of heavy aggregation, sample turbidity only increases noticeably when a significant fraction of the total protein has aggregated into large particles.

β-Lg and αCN contain 2 tryptophans, while βCN and αCN both only have one. The fluorescence ratio (F350/F330) at 20 °C (Fig. 2 I-I) was found to depend on the protein concentration and Ca2+ concentration in the following order: αCN > βCN > αCN > β-Lg (Fig. 2 I-I). The different fluorescence ratios observed at different protein concentrations under otherwise identical Ca2+ concentrations can be attributed to different relative populations of monomer and micelles. In contrast, β-Lg remains in the dimeric state even at higher concentrations, and thus the initial fluorescence ratio at 20 °C was found to be concentration-independent.

The fluorescence ratio can often be used to determine the fraction of unfolded protein at any point during the unfolding process. Environmental conditions such as temperature and the presence of Ca2+ can affect the protein aggregation, which will change the fluorescence of the proteins. Thus when evaluating the structural aspects of protein aggregation at different temperatures and Ca2+ concentrations, two factors are important, i.e. the change in the temperature and concentration and the spatial proximity of the proteins in micelles and/or aggregates, which is equivalent to an increase in their concentration (Faizullin, Konnova, Haertlé, & Zuev, 2013). Turbidity also can interfere with fluorescence measurement by causing light reflection and/or absorption. The dependency of unfolding and size of the proteins on Ca2+ at 20 °C shows the importance of recording the solution homogeneity and aggregation propensity simultaneously with fluorescence to improve the accuracy of the measurement. As summarized in Fig. 3a, by using combined differential scanning fluorimetry and dynamic light scattering, we showed that all properties of αCN measured at 20 °C before heating, were influenced considerably by Ca2+, while only the size and polydispersity of αCN and only polydispersity of β-Lg were affected significantly by Ca2+ (indicated by dark colors in Fig. 3a). The number of Ca2+ binding amino acids (phosphoserines), plays an essential role in this observation at 20 °C, where the extent of the effect of Ca2+ on JCN and αCN is highly dependent on the protein/Ca2+ ratio and consequently on the stoichiometry.

### 3.2. Aggregation tendencies of CNs and β-Lg at high temperatures

In the next step, the aggregation tendency and unfolding of the proteins during heating to 95 °C followed by cooling to 20 °C was evaluated by measuring fluorescence ratio at 350 and 330 nm, turbidity, hydrodynamic radius (Fig. 1 and Figs. S1–S3), Tsize (Fig. 2 m-p), and Tagg (Fig. 2 q-t). Tsize obtained by DLS exhibits the tendency of proteins to aggregate and/or assemble, which can correlate with thermal unfolding of proteins.

The data show a strong dependency of Tsize and Tagg for αCN on both Ca2+ and protein concentration, while those of βCN were highly dependent on the Ca2+ concentration and only weakly on the protein concentrations as indicated by PCA (Figs. S5 and S6). Tsize and Tagg of αCN and β-Lg were slightly dependent on the Ca2+ and protein concentrations. We found a larger variation in the Tagg (Fig. 2 q-t) of the CNs compared to the dependency of Tsize on the CNs and Ca2+ concentrations. The Tagg of αCN, JCN, and β-Lg is the same or slightly higher than their Tsize, which shows that the formation of the first aggregates immediately leads to the formation of very large particles. In terms of αCN, at most protein and Ca2+ concentrations, Tagg was higher than Tsize which indicates that αCN micelles assembled successively into ever larger particles. Although the initial size of αCN and β-Lg at 20 °C was less affected by Ca2+ than that of JCN and αCN, both protein and Ca2+ concentrations influenced the final aggregate size of all CNs and β-Lg at 95 °C (Fig. 1 and Figs. S1–S3), but to different extent depending on the protein. The final size of αCN, JCN and β-Lg depended on both protein and Ca2+ concentrations, while the αCN concentration had only slight effect on the final size.

The effect of Ca2+ on the assembly of the CNs increased with increasing temperature, which is in agreement with earlier findings (Dauphas et al., 2005; Li et al., 2019). JCN and αCN were more affected by Ca2+ at both low and high temperatures, due to the PS groups, but at higher temperatures Ca2+ also affected αCN, probably due to the interaction with carboxylate groups and shielding of negative charges. Temperature seems to be more effective than Ca2+ in shifting the equilibrium toward micelles and aggregates by increasing the hydrophobicity and monomer density in the micelles (Fox & McSweeney, 2013).

The unfolding transition of proteins causes significant changes in the compactness of the proteins or induces protein aggregation (Duy & Fitter, 2006), which can affect the red shift in the tryptophan fluorescence emission spectrum observed during unfolding. Duy and Fitter showed that the red shift caused by the unfolding of α-amylas was influenced by the aggregation of the protein (Duy & Fitter, 2006). For β-Lg, under conditions where turbidity did not measurably increase, i.e. 0.05 and 0.1 mM β-Lg in the absence of Ca2+, the fluorescence ratio increased without reaching a plateau until 95 °C and then decreased during subsequent cooling (Fig. S1). However, it did not reach the initial value, meaning that part of the protein molecules remained unfolded or associated in small aggregates. At higher β-Lg concentrations in the absence of Ca2+ and all protein concentrations in the presence of Ca2+, both turbidity (E-Tur) and fluorescence ratio reached a plateau almost at the same temperature, indicating that all of the proteins were unfolded and aggregated irreversibly as both the fluorescence ratio and turbidity were unchanged even after cooling to 20 °C (summarized in Fig. 3b). Indeed, in PCA, what characterizes the PCI of β-Lg responses is E-Tur showing a positive correlation to the increase in its protein concentration (Fig. S6), rather than to the change in the Ca2+ concentration (Fig. S5). Following the Le Chatelier’s principle (Atkins & De Paula, 2013), the aggregation of the unfolded proteins will cause the folding equilibrium to shift towards the unfolded state. The high and slight dependency of respectively E-Tur and of Tagg of β-Lg on Ca2+ confirms that Ca2+ has no effect on the unfolding of β-Lg and only induces the aggregation of unfolded β-Lg. Our finding is in accordance with results by Peixoto et al., where they showed that Ca2+ ions only bind to unfolded β-Lg at specific sites which might be a necessary feature to form the aggregates (Peixoto, Trivelli, André, Moreau, & Delplace, 2018).

The CNs behave differently from β-Lg during the heating and cooling steps. The profile of turbidity and the fluorescence ratio of αCN and βCN appeared more symmetrical over the heating and cooling steps, showing formation of reversible protein assemblies at a high temperature which dissociate upon cooling (summarized in Fig. 3c–e). However, the temperature profile of the radius was not reversible which could be due to bias in DLS caused by the presence of low numbers of stable large particles. The change in the fluorescence ratio of αCN during the heating process is more pronounced than for βCN dependent on both protein and Ca2+. The temperature profile of the fluorescence ratio during heating to 95 °C at different αCN and βCN concentrations was very similar, starting with a slight increase and then decreasing during heating to 95 °C; however, the cooling profile of αCN is not similar to its heating profile and the turbidity remains virtually constant at a high level even after cooling, which confirms irreversible formation of large aggregates at high temperatures, possibly due to disulphide bond formation (summarized in Fig. 3e).

Very little change in the secondary structure of JCN and αCN, as assessed by CD spectroscopy, suggests that the self-association of JCN and αCN even at high ionic strength and temperatures, did not cause
significant change in their structural conformation, which is also consistent with previous results (Farrell, Qi, Wickham, & Unruh, 2002; Li et al., 2019). However, whilst the molecular structure appeared to be unchanged, their mean aggregate size was influenced by both Ca\(^{2+}\) and temperature, and increased probably due to Ca\(^{2+}\) bridges between otherwise unchanged micelles.

3.3. The association of CNs and β-Lg

It is known from previous studies that CNs can have a chaperone-like behavior and reduce aggregation of proteins (Matsudomi et al., 2004; Yousefi et al., 2009). We first studied the interactions between the CNs and β-Lg at different temperatures. Then, in order to better understand the interaction of β-Lg and CNs in the presence of Ca\(^{2+}\) at 95 °C, we will occur during milk pasteurization for yogurt manufacture, the protein mixtures were heated at 85 °C for 15 min and analyzed with different experimental methods. Visual observation of the protein mixtures are shown in Fig. S11.

3.3.1. Combined differential scanning fluorimetry and dynamic light scattering

At 95 °C, in the absence of Ca\(^{2+}\), the size of the complex and turbidity depend on the CN concentration (Fig. 4a and b). By increasing CNs from 0.025 to 0.5 mM, the size and turbidity decreased, thus the CNs suppressed the aggregation of β-Lg.

Turbidity data (Fig. 4d) show that in the presence of 1 mM Ca\(^{2+}\), CNs did not significantly prevent aggregation, but according to the DLS data (Fig. 4b), CNs- β-Lg associations led to the formation of smaller aggregates in the size range of pure CNs.

The fluorescence ratio and turbidity data showed that none of the assemblies formed between CNs and β-Lg was reversible after cooling to 20 °C, similarly to the irreversible aggregation observed of β-Lg and κCN alone (Figs. S8–S10).

3.3.2. Circular dichroism (CD)

CD data of 15 min heated β-Lg mixed with CNs at 85 °C in the presence of 1 mM Ca\(^{2+}\) at different molar ratios were collected (Fig. S12). β-Lg contains α-helix and β-sheet structures (Liyanaarachchi & Vasiljevic, 2019), while CNs lack defined secondary structures due to high proline content in their primary structure (Fox, 2008). The more random and unordered structures of the heated proteins at lower CNs/β-Lg ratios (Fig. S12e) may stem from formation of unstructured and unfolded proteins, while α-strand structures may arise by intermolecular hydrogen bonded anti-parallel β-sheet structures during protein aggregation (Clark, Saunderson, & Suggett, 1981). Higher CNs/β-Lg ratios still resulted in heat aggregation and denaturation of β-Lg but to a lesser extent as indicated by higher preserved helical structure and less random structures (Fig. S12d). The CNs can act in a number of different ways to bring about the reduction in size. According to the fluorescence ratio and CD data and previous reports (Kehoe & Foegeding, 2011), CNs slightly affect denaturation of β-Lg in the heating process, but the decrease in turbidity and size of the complex in the absence of Ca\(^{2+}\) and only the size of the aggregates in the presence of Ca\(^{2+}\) would be obtained mainly through the interaction between the micelles and denatured β-Lg.

It seems that the electrostatic interactions in addition to the amphiphilic nature of αCN and βCN are responsible for their chaperone-like activity (De & Rotello, 2008; Kehoe & Foegeding, 2011). However, for the interactions with κCN, the initial stages could be due to the electrostatic and hydrophobic interaction, which reduces the molecular proximity at the level (1–2 Å) required for disulphide bond formation (Haque & Kinsella, 1988) involved in the κCN - β-Lg complexation (McKenzie, Norton, & Sawyer, 1971), suggesting that κCN interacted with intermediate species of denatured β-Lg.

3.3.3. Size exclusion chromatography (SEC)

We compared the retention volume of CNs in SEC with globular protein standards (Fig. S13). The molecular weight of analyzed peaks for all samples is higher than of the protein monomers, demonstrating that
they were multimeric and no detectable monomer population was found in the solution. Two complexes were observed in the $\beta$-Lg samples mixed with $\alpha$CN or $\beta$CN and both were larger than the protein monomers, while only one population was detected for the $\beta$-Lg mixed with $\kappa$CN. The protein bands for $\beta$-Lg and the CNs are also shown in the inset of Fig. S13.

### 3.3.4. Small angle X-ray scattering (SAXS)

Fig. 5 represents an overview of the background-subtracted SAXS scattering pattern of protein mixtures. SAXS analysis of $\beta$-Lg mixed with $\alpha$CN (a), $\beta$CN (b), and $\kappa$CN (c) before and after heating at 85°C for 15 min. Red line: sum of scattering of individual proteins before mixing and without heating, green line: mixtures before heating, black line: mixtures after heating. (d) Comparison of the scattering of the mixtures after heating. Fitting of SAXS data of mixtures of $\beta$-Lg with $\alpha$CN (e), $\beta$CN (f), and $\kappa$CN (g) after heating at 85°C for 15 min with a plugin model combining an ellipsoidal shape (protein structure) and polydisperse spherical model. The parameters are listed in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 6. Schematic representation of the titration of CNs by aggregated $\beta$-Lg in the presence and absence of $\text{Ca}^{2+}$ (a). Integrated peak of ITC enthalpogram of 1 mg mL$^{-1}$ of $\alpha$CN (b), $\beta$CN (c), $\kappa$CN (d) in 10 mM HEPES pH 7.0 in the absence and presence of 1 mM CaCl$_2$ titrated by 20 mg mL$^{-1}$ $\beta$-Lg at 85°C.

3.3.4. Small angle X-ray scattering (SAXS)

Fig. 5 represents an overview of the background-subtracted SAXS data of heated (H) $\beta$-Lg mixed with CNs. The overlapped summed scattering data of unmixed and unheated (UH) CNs and $\beta$-Lg ($\beta$-LgUH + CNsUH, red curve) and those of UH but mixed CNs and $\beta$-Lg (CNs and $\beta$-Lg) UH, green curve) for the three CNs may confirm that there are no aggregates formed between the CN and $\beta$-Lg at room temperature, as they exhibited almost similar scattering curves in the low-q and intermediate-q range (0.004–0.02 Å$^{-1}$). DLS data also confirmed that no detectable aggregates were formed after mixing the CNs with $\beta$-Lg at room temperature.
renders the binding interaction enthalpically favourable.

**Fig. 7.** Chaperone-like effects of CNs to reduce the aggregation of \(\beta\)-Lg. CNs reduce the aggregation of \(\beta\)-Lg through interaction with unfolded \(\beta\)-Lg. The aggregates formed between \(\beta\)-Lg and the CNs at high temperature are stable and do not dissociate upon cooling.

The presence of \(Ca^{2+}\) modulates the stoichiometry and strength of the interactions inferred from the enthalpograms. For all CNs and at all \(\beta\)-Lg concentrations, we observed a significant change in the interactions, which can be due to either a change in the energetic character of the interaction or a change in stoichiometry, or both, in the presence of \(Ca^{2+}\). The increase in the enthalpy of the interactions between \(\beta\)-Lg and \(\alpha\)CN due to the presence of \(Ca^{2+}\), is more pronounced than for \(\beta\)-Lg and \(\beta\)CN and \(\kappa\)CN, which can be due to the higher sensitivity of \(\alpha\)CN to the presence of \(Ca^{2+}\). From the enthalpy data, we can also conclude a higher enthalpy change due to the interaction of \(\beta\)-Lg with \(\kappa\)CN than with \(\alpha\)CN and \(\beta\)CN.

4. Conclusion

While the effect of heating and \(Ca^{2+}\) on the chaperone activity of CNs for \(\beta\)-Lg has been studied before, there are many open questions yet to be addressed. Here, we used a combination of different experimental approaches to show that the CNs and \(\beta\)-Lg behave differently at different temperatures and \(Ca^{2+}\) concentrations. While heat treatment affects denaturation and aggregation of \(\beta\)-Lg with little dependency on \(Ca^{2+}\), \(\alpha\)CN and \(\beta\)CN were influenced considerably by \(Ca^{2+}\). By comparing the present results with previous studies, we conclude that CNs display chaperone activity through slight suppression of \(\beta\)-Lg unfolding and that this process is also dependent on the type of CNs. Furthermore, the chaperone activity of CNs significantly limits the aggregate formation of unfolded \(\beta\)-Lg in the absence of \(Ca^{2+}\), or decreases the aggregate size in the presence of \(Ca^{2+}\), through hydrophobic interactions (Fig. 7). Our findings thus elucidate the molecular understanding of complexation of CNs and \(\beta\)-Lg and contribute to the wider discussions concerning the assembly of milk proteins to provide milk-based products with different functionalities.

**CRediT authorship contribution statement**

Hossein Mohammad-Beigi and Wahyu Wijaya: Conceptualization, Investigation, Writing Original draft, Writing - Review & Editing, Visualization. Mikkel Madsen, Yuya Hayashi, and Ruiwen Li: Investigation, Writing - Review & Editing. Tanja Christine Jæger, Tijs Albert Maria Rovers, Alexander K. Buell, Jacob J.K. Kirkensgaard, and Peter Westh: Conceptualization, Writing-review & Editing. Anna Bygvrå Hougaard: Project administration, Writing – review & Editing. Richard Ipsen: Conceptualization, Writing-review & Editing, Funding acquisition. Birte Svensson: Conceptualization, Supervision, Validation, Writing, Review & editing, Funding acquisition. All authors discussed the results, commented on the paper, and gave approval to the manuscript’s final version.

**Declaration of competing interest**

None. Note that Tijs Albert Maria Rovers is an employee of Arla Foods A.m.b.a. and Tanja Christine Jæger is an employee of Arla Food Ingredients Group P/S.

**Data availability**

Data will be made available on request.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2022.108373.


