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

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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 Ca^{2+} concentrations in a multi-parametric approach. While β-Lg was affected significantly through denaturation and resulting aggregation by heat treatment with little dependency on Ca^{2+}, αCN and βCN were influenced considerably by Ca^{2+}. 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 Ca^{2+} 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 Ca^{2+} 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, & Agnely, 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

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Table 1
Parameters of fitting of SAXS data with a plugin model combining an ellipsoidal shape (protein structure) to 0.07 to 0.2 Å and a polydisperse spherical model to lower q range from 0.005 to 0.07 Å.

Protein Mixture	A_radius (nm)	A_scale * 104	B_scale * 104	B_radius_polar (nm)	B_radius_equatorial (nm)
αCN+β-Lg	29.6	1.5593	1.3777	3.89	3.89
βCN+β-Lg	16.4	3.1178	1.0191	1.95	4.21
κCN+β-Lg	19.6	5.5756	0.54	4.41	9.06

(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 (α_{S1}-, α_{S2}-, 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 α_{S1}, α_{S2}, 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 secondary 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, Khalfin, & 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. α_S- 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). α_{S1}CN and βCN lack cysteine, whereas α_{S2}CN and κCN contain half-cystine residues engaged in intermolecular disulphide bonds and association with β-Lg (Fox, 2008).

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 (Uhrinova et al., 2000), and undergoes irreversible changes at temperatures between 70 and 140 °C (Iametti, 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, Claeys, & 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 disulphide 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 moiety 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, Otte, Ipsen, & Qvist, 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,

035 $M^{-1}cm^{-1}$, 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 $CaCl_2$. For the experiments at 85 °C, molar ratios of β-Lg/αCN, β-Lg/βCN, β-Lg/κCN at 1:0.04, 1:0.2, 1:0.4, 1:0.8 were prepared at fixed β-Lg and $CaCl_2$ 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 $CaCl_2$ 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 environment of the aromatic residues in the proteins. DLS was measured with a laser at 405 nm and turbidity with back reflection optics in the same capillaries. The experiments were performed by scanning from 20 to 95 °C and then cooling to 20 °C at a scan rate of 1.5 °C/min. The instrument software automatically determined the temperature of aggregation onset (T_{agg}) and cumulant radius. To determine the onsets of turbidity (aggregation onset) the software looks for the first two-state transition in the data. It then uses a linear fit on the baseline and a two-state fit on the transition. The onset corresponds to the temperature at which the two-state model fit deviates from the linear fit (baseline) by more than 0.5%. To calculate the onset of the cumulant radius increase (T_{size}) by the software, first, the initial radius or initial scattering intensity is calculated by a median fit to the initial data points (baseline). Second, the point of the curve is determined at which the radius or scattering intensity is increased by 10% (i.e. by a factor of 1.1). Third, the slope of the curve at this point is prolonged towards the baseline and the onset corresponds to the point, at which the slope intersects the baseline.

2.3. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was performed using the NanoITC2G (TA Instruments, Delaware, USA). For binding of Ca^{2+} to milk proteins, the sample cell was filled with 0.05 mM β-Lg, αCN, βCN, κCN in 10 mM HEPES pH 7.0 (1000 μL), and the syringe with 50 mM $CaCl_2$ (250 μL). Binding between β-Lg and CNs at 85 °C was observed by titrating 0.05 mM CNs in 10 mM HEPES pH 7.0 in the absence and presence of 1 mM $CaCl_2$ with 30 injections of 6 μL of 1 mM β-Lg in the same buffer, each with a duration time of 2 s and a spacing time of 300 s. The solution in the cell was continuously stirred at 250 r.p.m. Heats of dilution were subtracted from the titration data using relevant control titrations.

2.4. Circular dichroism (CD) spectroscopy

CD spectroscopy was performed using a Jasco J-1500 spectropolarimeter (JASCO, Inc., Tokyo, Japan). The CD spectra of the β-Lg/βCN complexes were recorded between 190 and 250 nm at 25 °C, by scanning with a speed of 100 nm/min, 2 s response time, and 1 nm step size. β-Lg/βCN mixtures were diluted 10 times using deionized water. 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/βCN 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 ÄKTA 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% CH_3COOH, 50% CH_3OH, and 40% H_2O. 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/αCN, β-Lg/βCN, β-Lg/κCN were prepared by diluting and mixing protein solutions in 10 mM HEPES pH 7.0 containing 1 mM $CaCl_2$ 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 $\lambda = 0.154$ 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 $q = 4\pi\sin\theta/\lambda$ 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:

$$I(q) = \Delta\rho^2 n_p V_p^2 P_p(q) S(q)$$

where $\Delta\rho^2$, n_p, 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

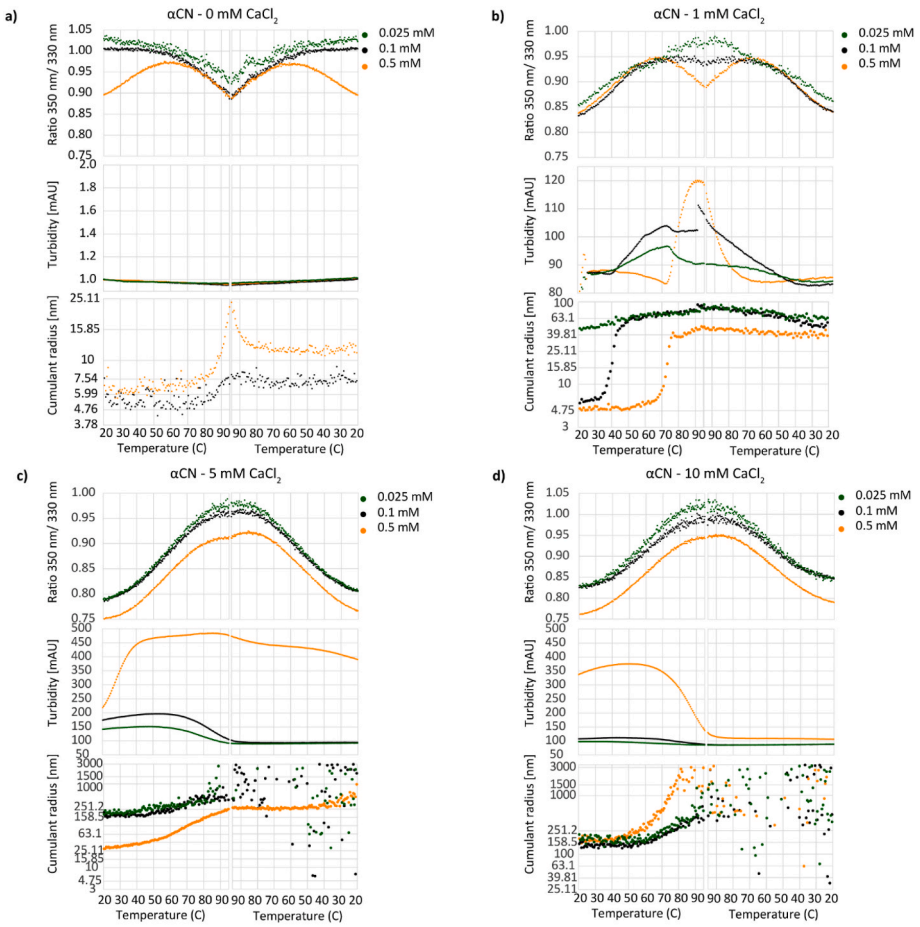


Fig. 1. Thermal stability and aggregation of αCN. Thermal unfolding followed by intrinsic fluorescence, turbidity, and dynamic light scattering of αCN (a) in the absence and in the presence of (b) 1 mM, (c) 5 mM, and (d) 10 mM $CaCl_2$ was measured by scanning in the range 20–95 and then 95-20 °C at 1.5 °C/min.

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):

$$P(q;\alpha) = \frac{scale}{V} F^2(q;\alpha) + background$$

where.

$$F(q;\alpha) = \nabla\rho V \frac{3(\sin(qr) - qrcos(qr))}{(qr)^3}$$

and.

$$r = \left[R_e^2 \, sin^2\,\alpha + R_p^2 \, cos^2\,\alpha\right]^{\frac{1}{2}}$$

α is the angle between the axis of the ellipsoid and q, and $V=(4/3)\pi R_p R^2$ is the volume of the ellipsoid, Rp is the polar radius along the rotational axis of the ellipsoid, Re 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, Ahrne, & Kirkensgaard, 2021).

$$P_{PS}(q) = \int P_S(q) D(R) V(R)^2 dR$$

where the monodisperse sphere form factor is given by.

$$P_S(q) = \left[3\frac{(\sin(qR) - qRcos(qR))}{(qR)^3}\right]^2$$

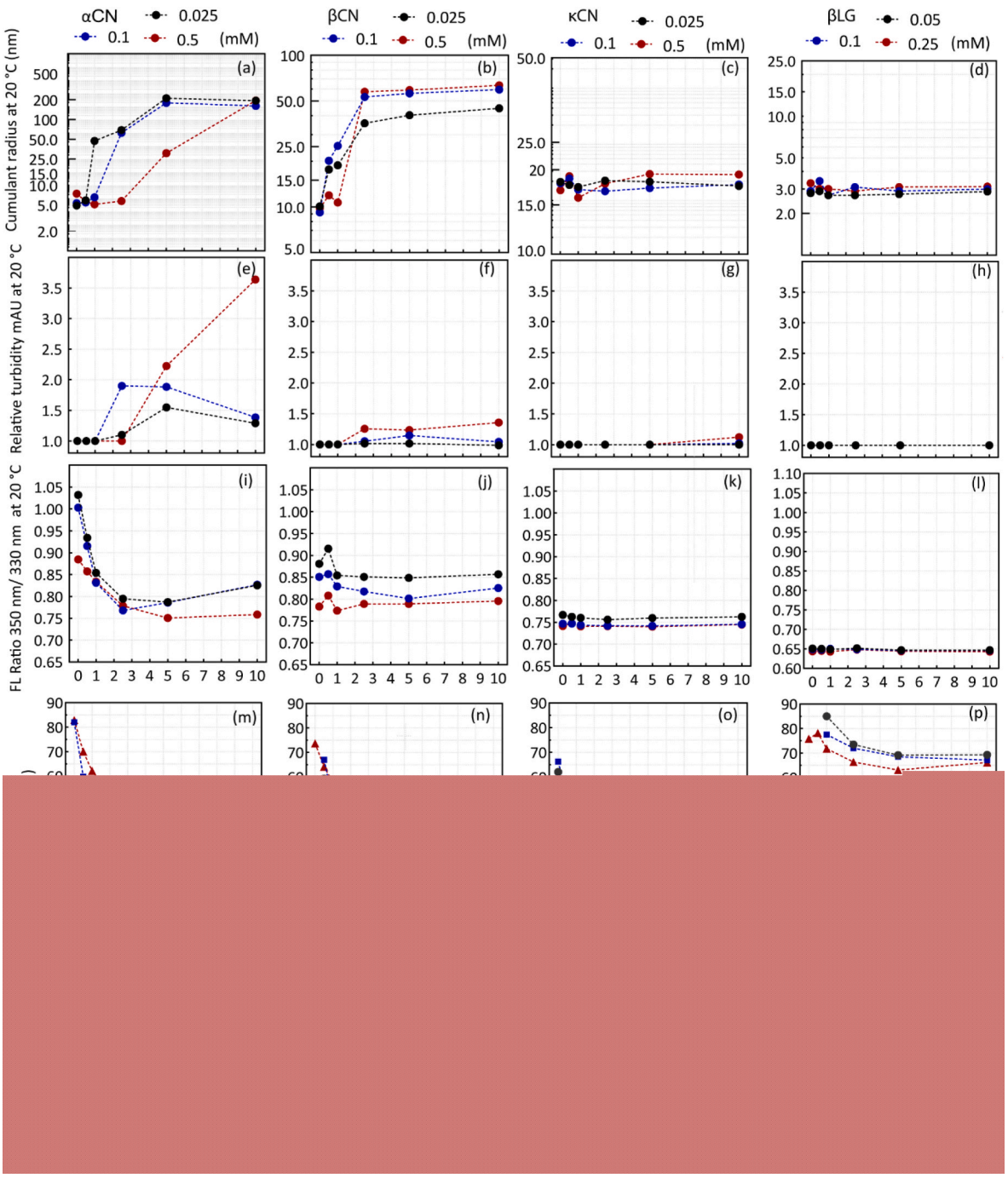
2.8. Principle component analysis

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 (T_{agg}, 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) (Lê, 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



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% ; 3 0E0K;

& 5 7<7@$ 5E,@ 6, * * 5 '26
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F<', % 1.10 ,8 5/ # 6
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