Effect of temperature, calcium and protein concentration on aggregation of whey protein isolate: Formation of gel-like micro-particles

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Abstract
Protein aggregation occurs in biological systems and industrial processes, affecting protein solubility and functional properties. In this study, whey protein isolate (WPI) obtained from bovine milk was used as a model to study the dependence of aggregation on pre-heating temperature and on protein and calcium concentrations. WPI solutions (0.1–5.0%, w/v) were heated at 25–85°C for 30 min prior to cooling and calcium addition. Tryptophan shifted to a more hydrophilic environment as WPI concentrations and pre-heating temperatures increased. Pre-heated WPI solutions yielded soluble particles, which aggregated to form porous gel-like particles by addition of calcium chloride. WPI microgel particles could be prepared by using a cold gelation method and preheated the protein above 65°C. The particle size was mono-disperse with sizes of about 190 nm and 255 nm, respectively in solutions pre-heated to 75 or 85°C and containing 5 mM calcium.

1. Introduction

Protein aggregation is a phenomenon that occurs both as a part of normal biological functions and as an aberrant process in deleterious conditions such as neurodegenerative disease (Morris, Watzky, & Finke, 2009). It is also a common and troublesome manifestation of protein instability encountered during fermentation, refolding, shearing, agitation, freeze-thawing, drying, reconstitution and storage processes in the development of protein-based drugs (Wang, 2005). Studies of protein folding are usually complicated by aggregation of intermediate and denatured states (Bondos & Bichnell, 2003). Protein aggregation is a major topic in the field of food science, and its regulation is believed to affect the texture of protein-enriched products (De Jongh & Broersen, 2012). The protein aggregation problem is thus common to biological systems, scientific research, and medical or industrial applications (Bondos & Bichnell, 2003). Intrinsic molecular structural factors and extrinsic factors including concentration, general solution conditions, container/closure system and surfaces, light and irradiation all contribute to protein aggregation (Wang, Nema, & Teagarden, 2010). It is important to understand the aggregation process of a variety of proteins to prevent it or exploit it for applications.

The textural functionality of a protein-based food product is determined to a large extent by the properties of micro-structural elements and their mutual interactions. These micro-structural aspects stem from the aggregation behaviour of the protein, which in turn is determined by molecular properties and interactions. Bovine milk proteins contain about 80% casein and 20% whey proteins, a mixture of globular proteins. Heat-induced aggregates of κ-casein and denatured whey proteins formed in milk-based dairy mixtures (Guyomarch, Law, & Dalgleish, 2003). Whey proteins denatured, aggregated and associated with the casein micelles during preheating, evaporation and spray drying related to milk powder manufacture (Oldfield, 1996). In an attempt to increase whey protein levels in cheese, depending on the sizes of whey protein aggregates, their addition to milk decreased the strength and contraction capacity of rennet-induced gels but increased curd moisture content (Giroux, Lanouette, & Britten, 2015).

Food proteins are used widely in formulated foods since they have high nutritional value and possess unique functional properties.
properties including gelation, emulsification, foaming, and ligand-binding capacity (Chen, Remondetto, & Subirade, 2006; De Wolf & Brett, 2000). Their functional properties depend somewhat on the extent of denaturation or aggregation (Ako, Nicolai, & Durand, 2010; Nicolai, Britten, & Schmitt, 2011). Cold-induced gelation of globular proteins can be achieved by heat-induced unfolding to form soluble particles, followed by formation of a network via cation-mediated or acid-induced aggregation at ambient temperature (Alting, Hamer, De Kruif, & Visschers, 2003; Barbut & Foegeding, 1993). Pre-heating treatment was required to induce cold gelation and performed above protein denaturation temperature and below the critical concentration for heat-induced gelation at neutral pH and low ionic strength conditions (Hongsprabhas & Barbut, 1998; Mudgal, Daubert, & Foegeding, 2011). Formation and structure of protein self-supporting gels vary with protein and cation concentrations (Remondetto & Subirade, 2003).

It is interesting to develop nanoparticles (0.5–50 nm in radius) and microparticles (50–500 nm) for encapsulation and protection of sensitive and bioactive ingredients, which may reduce the impact of encapsulated ingredients on food texture and to improve functional ingredient delivery. The particles based on denatured whey protein and with the radius ranged from 50 to 150 nm have been prepared by pH-cycling treatment and use of calcium during particle formation could control particle compactness (Giroux, Houde, & Britten, 2010). Cold-set whey protein gels have a porous inner structure (Kunn, Cavallieri, & da Cunha, 2010). Below a critical protein concentration of gel formation, stable suspensions of polydisperse aggregates may be produced (Ako et al., 2010). Development of high-surface-area protein nano/micro-particles might provide new opportunities for pulmonary and transdermal delivery applications (Engstrom, 2007).

In this study, whey protein isolate (WPI) obtained from bovine milk was used as a model to study the dependence of protein aggregation on pre-heating temperature and on protein and calcium concentrations. The potential for obtaining cold-set gel-like nano/microparticles from the apparent absorbance measured at 500 nm using a Mapada UV-1200 UV–Visible spectrophotometer (Shanghai Mapada Instruments Co. Ltd, Shanghai, China).

2. Materials and methods

2.1. Materials

WPI (BioPRO, ~92% protein) was obtained from Davisco International Inc. (Le Sueur, MN, USA). Pyrene (~99.0%, for fluorescence) and 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS, ~97.0%, for fluorescence) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Other reagents were analytical grade and obtained from SinoPharm CNCM Ltd. (Shanghai, China).

2.2. Sample preparation

WPI powder was dispersed in distilled water by gentle stirring for 2 h at room temperature to ensure complete hydration of the protein, followed by adjusting to pH 7.0 using 0.1 mol L⁻¹ NaOH or HCl. The solution was adjusted to a protein concentration of 6.00% (w/v), which was calculated without considering the purity of WPI, and then diluted with the distilled water at pH 7.0 to a WPI concentration of 0.1, 0.25, 0.5, 1.0, 2.0 or 5.0%. The solutions were then held at a temperature from 25°C to 85°C for 30 min after reaching the temperature and then cooled and held at room temperature (~25°C) for 2 h (Leung, Sok Line, Remondetto, & Subirade, 2005). WPI/CaCl₂ mixtures were prepared by mixing pre-heated protein solutions at 0.12, 0.3, 0.6, 1.2, 2.4 or 6.0% and CaCl₂ solutions at a 5:1 volume ratio under gentle stirring for 1 h, to obtain a WPI concentration of 0.1, 0.25, 0.5, 1.0, 2.0 or 5.0% and a CaCl₂ final concentration of 1.7, 5 or 10 mM. All samples were prepared at least in duplicate for each subsequent analysis.

2.3. Fluorescence measurements

Steady-state fluorescence was measured using a Fluoro Max 4 fluorescence spectrophotometer (Horiba Jobin Yvon Inc., Edison, NJ, USA), equipped with 10 mm quartz cuvettes. Fluorescence of WPI was recorded with an excitation wavelength of 295 nm. Fluorescence intensity was normalised relative to the emission maximum (λmax) peak for 0.1% WPI at 25°C. Pyrene and ANS were added as fluorescence probes at final concentrations of respectively 1.0 and 19.8 μM. The solutions were then kept at room temperature overnight. Pyrene fluorescence at an excitation wavelength of 335 nm was recorded from 350 to 450 nm and I/I₁ (ratio of the intensities of the first and third band of the emission spectra) was calculated. The fluorescence emission spectrum of ANS excited at 370 nm was recorded from 390 to 650 nm. The spectral resolutions are 1.5 and 2.0 nm, respectively, for protein intrinsic fluorescence and pyrene or ANS fluorescence for both excitation and emission.

2.4. Turbidity measurement

Turbidity (% transmittance) of WPI solutions was determined from the apparent absorbance measured at 500 nm using a Mapada UV-1200 UV–Visible spectrophotometer (Shanghai Mapada Instruments Co. Ltd, Shanghai, China).

2.5. Size and ζ-potential measurements

Particle size distribution and ζ-potential were measured on a Zetasizer Nano ZS90 particle analyser (Malvern Instruments Ltd, Malvern, UK) with a He/Ne laser (λ = 633 nm). All measurements were conducted at 25°C and at a scattering angle of 90°. For dynamic light scattering measurement, samples were prepared with the distilled water (pH 7.0) which was filtered through a 0.22 μm pore membrane.

2.6. Statistical analysis

Data were analysed for significant differences using the online GraphPad QuikCalc Free t-test calculator (GraphPad Software Inc. San Diego, CA, USA).

3. Results and discussion

3.1. Impact of whey protein isolate concentration and temperature

3.1.1. Structural sensitivity of whey protein isolate

Intrinsic fluorescence has been used widely to investigate protein tertiary structure and structural transitions (Bhattacharjee & Das, 2000; Halder, Chakraborty, Das, & Bose, 2012; Liang, Yao, & Jiang, 2005). Table 1 shows λmax and the λmax intensity of WPI fluorescence as a function of concentration after pre-heating at various temperatures. In the case of 25°C treatment and 0.1% WPI, λmax was around 335 nm, indicating that tryptophan residues are in a hydrophobic environment. The λmax changed very little as the concentration increased, reaching 338 nm at 5.0%. The spectral intensity increased with concentration up to 0.25% and then began to decrease. The changes in the fluorescence λmax and intensity were similar for pre-heating at 25 and 45°C. The overall trend was similar at 65°C except that intensity at a given concentration was a bit greater and λmax reached 339 nm at 5.0% WPI. The increases in
intensity were larger in the higher temperature cases. At 75 °C and 85 °C, λ\text{max} shifted from 336 nm and 337 nm at 0.10% to longer wavelengths at higher WPI concentrations, reaching about 345 nm at 5%. The red shift indicates that tryptophan was in a more hydrophobic environment, suggesting protein unfolding (Bhattacharjee & Das, 2000). These results indicate that the degree of WPI unfolding increased as its concentrations and pre-heating temperatures increased.

The major proteins in bovine whey are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), which account, respectively, for about 50% and 20% of the total protein content. Fluorescence emission spectra of β-LG and α-LA show λ\text{max} around 336 and 327 nm, respectively, in its native state (Liang, Zhang, Zhou, & Subirade, 2013). The fluorescence intensity of β-LG is greater than that of α-LA at the same molar concentration (Liang et al., 2013). The thermal unfolding of these proteins has been investigated thoroughly and their transition temperatures appear to be respectively about 73 °C and 66 °C (Hong & Creamer, 2002), varying slightly with protein concentration, pH, and ionic composition and concentration (Relkin, 1996). From the results in Table 1, it can be seen that a significant change in the WPI fluorescence was observed basically at the pre-heating temperatures of 75 °C and 85 °C. The fluorescence characteristics of WPI (Table 1) therefore appear attributable mainly to β-LG.

The reversibility of the structural transitions in the absence or presence of stresses is the subject of conflicting reports. Bhattacharjee and Das (2000) found that β-LG underwent an irreversible change in structure based on ANS fluorescence but a reversible change based on tryptophan fluorescence when heated to 90 °C followed by gradual cooling or to 70–90 °C for 15 min followed by cooling to 25 °C. It is noteworthy that the change in WPI structure was dependent on concentration when heated to 75 °C or 85 °C for 30 min followed by cooling to room temperature, based on tryptophan fluorescence (Table 1). The change in the fluorescence of WPI was reversible at 0.1% and 75 °C and 85 °C and at 0.25% and 75 °C but not at higher concentrations.

### 3.1.2. Whey protein isolate aggregation

The turbidity of a colloidal dispersion at a given wavelength depends on the particle number concentration, radius, and refractive index, as well as the refractive index of the medium (Jeffrey & Ottewill, 1988). The presence of protein aggregates or particles often results in an increase in the turbidity of the solution, which may thus be used to qualitatively assess the extent of aggregation (Wei & Polozova, 2013). The turbidity of WPI solutions increased with concentration at certain temperatures in the 25–85 °C range (Fig. 1A). Turbidity was not dependent on temperature, except that it increased at 85 °C and concentrations above 0.25%. These results suggest the aggregation of WPI increased as the protein concentrations increased and as the preheating temperature increased up to 85 °C. In view of similar results obtained at 25 °C and 45 °C...
WPI aggregation was not investigated at 45 °C in the experiments discussed below. The behaviour of WPI in solution was further analysed using pyrene fluorescence, which has been used widely to monitor the macromolecular association and micelle formation in solution (Li, Jiang, & Wu, 1997; Liang, Yao, Gong, & Jiang, 2004). The I1/I3 ratio was about 1.5 for pyrene in water (Liang, Pinier, Leroux, & Subirade, 2009). In WPI solutions at a concentration of 0.1%, the ratio decreased to about 1.16 at 25 °C and to about 1.14 at pre-heated temperatures of 65, 75 and 85 °C (Fig. 1B), indicating that most pyrene molecules transferred to a hydrophobic environment and that WPI aggregated to form colloidal particles. The ratio continued to decrease with increasing protein concentration, more so at lower temperatures, reaching 1.02 at 5.0% and 25 °C. At this concentration, the I1/I3 ratio was 1.05 at 75 °C and 85 °C. A slightly greater ratio suggests that WPI particles were more loose, possibly due to thermal unfolding that made the protein form more extended structure at 75 °C and 85 °C (Table 1). The pyrene I1/I3 ratios are not as low as 0.95 measured for bulk polystyrene (Li et al., 1997), suggesting that the core of the WPI particles still contained some hydrophilic groups and/or water molecules.

Fig. 2 shows size distributions of WPI particles at various concentrations in association with different heating treatments. At 0.10% and 25 °C, there are two size distributions, a major one around 64 nm and a minor one around 10 nm. Increases in WPI concentration did not affect the major size distribution to any appreciable degree. In the case of heating at 65 °C and 0.1%, the size distribution shows one peak ranging from 17 to 178 nm and averaging around 60 nm. As WPI concentrations increased, the size distribution did not change significantly, which shifted to about 85 nm at a WPI concentration of 5.0%, while a minor and smaller size distribution appeared. Only one smaller size distribution exists if the calculation is based on the number distribution at 25 and 65 °C (data not shown). At the lower WPI concentrations, the smaller particles might be not enough to detect by using the distribution by intensity since the intensity of scattering is proportional to the sixth power of the size according to Rayleigh’s approximation. Somewhat similar changes in size distribution were observed as the WPI concentration was increased at higher temperatures. In the case of heating at 75 °C and 0.10%, the size was around 31 nm, and then a minor and smaller size distribution around 6 nm appeared and the size distributions basically kept invariant until 1.0%, after which two peaks were around 10 and 86 nm. At 85 °C, the size was around 17 nm at 0.1% WPI and increased to about 20 nm at 1.0% and then became distributed to two peaks appearing around 9 and 133 nm at 5.0%. The change in the size distributions might be attributed to the protein different structure at various concentrations (Table 1). Together with turbidity and pyrene fluorescence, these results indicate that WPI aggregated to form soluble particles with the sizes from nano-to micro-scales under the investigated conditions.

The ζ-potential distribution of WPI particles shows a single peak under the investigated experimental conditions. Fig. 3A shows the change in the ζ-potential of WPI particles as a function of temperature. The isoelectric points of β-LG and α-LA are around pH 5.2 and pH 4.5–4.8, respectively (Almecija, Ibanez, Guadix, & Guadix, 2007). The ζ-potential of WPI particles was negative at pH 7.0. At 25 °C, it was −30 mV at 0.1% WPI and decreased as the protein concentration increased, reaching −21 mV at 5.0%. The ζ-potential increased as the pre-heating temperature was increased, reaching −46 and −30 mV at 0.10% and 5.0% and 85 °C, respectively. Generally, particles with absolute ζ-potential values above 30 mV are considered stable (Honary & Zahir, 2013). Negatively charged groups on the surface of WPI particles provide electrostatic repulsive forces between particles and stabilise the particles in solution. Moreover, due to the presence of steric stabilisation for high-

Fig. 2. Size distributions of whey protein isolate particles obtained at various concentrations of solutions pre-heated at (A) 25 °C, (B) 65 °C, (C), 75 °C, (D) 85 °C.
molecular-weight molecules, a $\zeta$-potential of 20 mV may provide sufficient stabilisation (Honary & Zahir, 2013).

As the WPI concentration increased, the intermolecular distance in solution decreased, causing an increase in protein molecule interactions (Figs. 1 and 2). It has been reported that formation of $\beta$-lactoglobulin dimers produces fluorescence self-quenching (Renard, Lefebvre, Griffin, & Griffin, 1998). Tryptophan fluorescence of $\beta$-LG was possibly quenched by the proximity of the Cys66–Cys160 disulphide bond, the Arg124 residue, the association site for dimer formation, and lysine amine (e-NH$_2$) groups (Bhattacharjee & Das, 2000; Croguennec, Molle, Mehra, & Bouhallab, 2004). Together with the fluorescence results, it is thus suggested that WPI aggregation was the principal reason for the decrease in protein fluorescence intensity above 0.25% (Table 1).

The size and structures of whey protein aggregates were dependent on the protein concentration and on the heating protocol. Heat denaturation exposed the buried hydrophobic residues in native state to the solvent phase, which is more pronounced as WPI concentrations increased (Table 1). WPI aggregated to form soluble particles containing hydrophilic groups and/or water molecules in the core (Fig. 1) and with the sizes from nano- to micro-scales (Fig. 2). At neutral pH, $\beta$-LG and $\alpha$-LA interacted to form mixed oligomers and aggregates by non-covalent bonds and disulphide bonds, leading to a lower critical association concentration in WPI than in pure $\beta$-LG solutions (Nicolai et al., 2011). WPI particles were stabilised by negatively charged groups on the surface (Fig. 3A).

3.2. CaCl$_2$-induced formation of gel-like whey protein isolate microparticles

The sensitivity of protein structure to the presence of salts is well known, and the influence of cations on protein aggregation during the formation of heat-set and cold-set gel has been studied widely. Addition of CaCl$_2$ before heat-induced gelation greatly affects the hardness of WPI gel at a protein concentration of 10% (Ju & Kilara, 1998). $\beta$-lactoglobulin at 6% formed two different microstructural gels called “filamentous gel” and “particulate gel”, depending on the cation concentration (Remondetto & Subirade, 2003). However, Ca$^{2+}$-induced aggregation at temperatures below the denaturation temperature and at protein concentrations below the critical concentration for cold-set self-supporting gel formation has not received much attention. In this study, it was found that addition of CaCl$_2$ caused an increase in WPI solution turbidity, with the most change at the salt concentrations between 0.5% and 2% (Fig. 4). A protein concentration of 1.0% was selected for further study of the influence of CaCl$_2$ on WPI aggregate and the potential of gel-like particle formation. The pyrene fluorescence I$_1$/I$_3$ ratio at this concentration was about 1.07, regardless of pre-heating temperature and CaCl$_2$ concentration.

Fig. 5 shows the size distribution of CaCl$_2$-induced aggregates of WPI at various temperatures. At 25 °C and 1.7 mM CaCl$_2$, there were three size categories, the major one being around 190 nm, which is larger than the size of pure WPI particles. Further aggregation was observed as the CaCl$_2$ concentration increased, with three major size distributions around 122 and 600 nm at 5.0 mM and around 106 and 1281 nm at 10.0 mM. At the same time, minor size distributions, which are smaller than that of pure WPI particles, were also observed. These results suggest that CaCl$_2$ induced simultaneous dissociation and aggregation of WPI particles. The phenomenon was also observed for milk-concentration proteins added calcium-chelating salts (Yang, Liu, & Zhou, 2012). In the case of heating at 65 °C, adding 1.7 mM or 5.0 mM CaCl$_2$ resulted in the appearance of a size distribution around 295 nm, suggesting the salts induced the aggregation of a portion of WPI particles. Increasing the CaCl$_2$ concentration to 10.0 mM led to the disappearance of two minor peaks around 10 and 60 nm, suggesting that the salt concentrations were high enough to interact with total WPI particles to form larger aggregates. For 75 °C, a similar change was observed but with a new peak appearing around 190 nm at 1.7 mM and disappearance of two minor peaks at 5.0 mM. For 85 °C, a major size distribution around 44 nm was observed with a shoulder around 255 nm at 1.7 mM CaCl$_2$, while a single 255 nm diameter aggregate category was observed at 5.0 mM CaCl$_2$. These results suggest that CaCl$_2$ induced WPI to form larger aggregates in the micro-sized scale at 65–85 °C. The monodisperse microparticles were formed at the highest concentrations of CaCl$_2$. The size distribution of CaCl$_2$-induced WPI aggregates at 10 mM CaCl$_2$ was not studied because of precipitation at 75 °C and gel formation at 85 °C. Fig. 3B shows the $\zeta$-potential of CaCl$_2$-induced WPI aggregates. The $\zeta$-potential of aggregates obtained at 1.7 mM CaCl$_2$ is significantly less for WPI particles obtained without CaCl$_2$ (Fig. 3A) for all pre-heating temperatures tested and continued to decrease with an increase in the CaCl$_2$ concentration used. In the 25 °C and 65 °C cases, the $\zeta$-potential was about −6 mV when the CaCl$_2$ concentration was increased to 10 mM. For 75 °C and 85 °C, the $\zeta$-potential
was about $-11 \text{ mV}$ at 5 mM CaCl$_2$. These results suggest that adding CaCl$_2$ neutralised negative charges on the WPI particle surface (Fig. 3A) thereby allowing intermolecular cross-linking and hence aggregation (Remondetto & Subirade, 2003). Moreover, it has been reported that ion-induced conformational changes and electrostatic shielding are involved in salt-induced protein aggregation (O’Kennedy & Mounsey, 2009).

ANS is a well-known fluorescence probe used for measuring protein surface hydrophobicity and characterising structure porosity during unfolding and aggregation (Bhattacharjee & Das, 2000; Kundu & Guptasarma, 2002). It has a low fluorescence quantum yield at $\lambda_{\text{max}} \approx 525 \text{ nm}$ in aqueous solution (data not shown). When ANS was added to WPI solution in the absence of CaCl$_2$, the $\lambda_{\text{max}}$ shifted to 470 nm in the cases of 25 °C and 65 °C treatments and to 466 nm for 75 °C and 85 °C, accompanied by a significant increase in fluorescence intensity (Fig. 6), indicating that ANS was bound to the hydrophobic regions of the whey protein molecules. For these four temperatures, the fluorescence intensity of ANS in WPI solution was about 237, 209, 198 and 248 times that of ANS in aqueous solution. Adding CaCl$_2$ did not affect the $\lambda_{\text{max}}$ but caused a change in fluorescence intensity. At 25 °C, a slight increase was observed but stayed constant as the calcium concentration increased from 1.7 to 10 mM. For 65 °C, the fluorescence intensity increased gradually with CaCl$_2$ concentration. A similar change with an overall more pronounced effect was observed at 75 and 85 °C. For the four temperature treatments, the ANS fluorescence intensity at 5 mM CaCl$_2$ was respectively about 1.07, 1.15, 1.48, 1.84 times that in WPI solution, suggesting that CaCl$_2$-induced WPI aggregates have more hydrophobic surface exposed for access by ANS molecules. The surface area of a particle generally increases as size decreases. Considering the results obtained for CaCl$_2$-induced aggregation of whey protein (Fig. 4), it may be concluded that the aggregates are porous. This result is consistent with the formation of a honeycomb inner structure in calcium-induced soy-protein-isolate nanoparticles by using a cold gelation method (Zhang, Liang, Tian, Chen, & Subirade, 2012). It is therefore conceivable that CaCl$_2$ caused the aggregation of WPI particles to form cold-set gel-like particles.

Heat-induced microgels with a radius varied between 75 and 150 nm were previously prepared using $\beta$-LG, $\alpha$-LA/$\beta$-LG mixtures (20/80, w/w), and whey protein isolate at a protein concentration of 4% and heating at 85 °C for 15 min at pH 5.7–6.2 (Schmitt, Bovay, Vuilliomenet, Rouvet, & Bovetto, 2011). Cold-set WPI gel particles below 50 μm in radius were prepared by an emulsification/internal gelation technique while cold-set WPI gel beads with the radius of 0.5–1 mm were produced by the external method involving dropwise addition of denatured WPI into a CaCl$_2$ solution (Egan, Jacquier, Rosenberg, & Rosenberg, 2013). In this study, WPI microgel particles with a monodisperse size at 190 and 255 nm were prepared by preheated the protein respectively at 75 and 85 °C and by using a cold gelation method. The cold-set WPI microgels could be used as an effective carrier of heat-sensitive and

![Fig. 4. Turbidity of whey protein isolate (WPI) solutions pre-heated at (A) 25 °C, (B) 65 °C, (C) 75 °C and (D) 85 °C in the presence of (■) 1.7 mM, (○) 5 mM and (▲) 10 mM CaCl$_2$, as a function of concentration. Data are the mean ± standard deviation.](image-url)
Fig. 5. Size distributions of whey protein isolate particles obtained at various CaCl$_2$ concentrations from solutions pre-heated at (A) 25 °C, (B) 65 °C, (C) 75 °C and (D) 85 °C.

Fig. 6. Fluorescence emission spectra of 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS) in suspensions of Ca$^{2+}$-induced whey protein isolate aggregates at various Ca$^{2+}$ concentrations (— 0 mM; —— 1.7 mM; —— 5 mM; —- —- 10 mM) at (A) 25 °C, (B) 65 °C, (C) 75 °C and (D) 85 °C.
bioactive ingredients and be added to food systems where their incorporation would be acceptable from a texture perspective.

4. Conclusions

The aggregation of WPI was dependent on pre-heating temperature and on protein and calcium concentrations. Pre-heated WPI solutions yielded soluble particles containing hydrophilic groups and/or water molecules in the core and stabilised by negatively charged surface groups. The WPI aggregation resulted in a decrease in the tryptophan fluorescence intensity at WPI concentrations above 0.25%. Calcium ions caused further aggregation of whey proteins, yielding particles that were porous and having a gel-like inner structure when formed from solutions pre-heated above 65 °C. These particles were mono-dispersed with a micro-scale size when the calcium concentration was 5 mM and the pre-heating temperature was 75 °C or 85 °C. It is thus concluded that WPI micro-particles could be prepared by using a cold gelation method.

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