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Heat induced conformational changes of whey proteins in model infant formulae: Effect of casein and inulin

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ABSTRACT

The effect of casein (CAS) and inulin (INUL), at ratios commonly found during infant formula processing, on the conformational changes of whey proteins (WP) was investigated. Model systems were prepared as aqueous dispersions of WP isolate alone or with calcium caseinate and/or INUL, and heated for 30 min at 66, 70 or 75 °C. Heat treatment of WP induced denaturation and aggregation. Solubility of WP system decreased by 25% when heated at 75 °C. Upon heating, the presence of CAS did not prevent WP denaturation, but increased protein solubility up to 100% in WP–CAS system and prevented aggregation. INUL affected the secondary structure of WP and increased the denaturation temperature, although it did not prevent aggregation nor solubility loss at 75 °C. In conclusion, presence of CAS prevented aggregation and solubility loss of WP during heating while INUL induced WP conformational changes impacting the denaturation stage.

1. Introduction

Infant milk formula (IMF) is used as a substitute for human milk when it is not possible to provide the infant with mother's milk. Cow and human milk have important compositional differences. Human milk has lower total protein content and higher whey protein-to-casein ratio (60:40) than cow milk (20:80). Carbohydrate composition also differs between milk from both species, having human milk a higher lactose concentration than bovine milk (Nasirpour, Scher, & Desobry, 2006).

Oligosaccharides are important components of human milk, both for the prebiotic effect that they exert and because they comprise the third largest fraction after lactose and fat, with a concentration of about 12–14 g L^{-1} (Coppa, Zampini, Galeazzi, & Gabrielli, 2006; Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015; Nasirpour et al., 2006). Therefore, cow milk needs to be transformed to produce a formulation that resembles the nutritional profile of human milk. The transformation includes modification of protein profile and content and addition of oligosaccharides. Galacto-oligosaccharides and long-chain fructo-oligosaccharides, or inulin with a degree of polymerisation higher than 10, are oligosaccharides commonly added during IMF production (Boehm, 2013; European Commission, 2016).

During IMF production, the ingredients are recombined, rehydrated and heat treated (Blanchard, Zhu, & Schuck, 2013). Whey proteins (WPs) are heat-labile and they can undergo conformational changes and loss of functionality during processing. β-Lactoglobulin, the main WP in cow milk, suffers denaturation when heated above 65 °C, exposing the free thiol group, which leads to aggregation with other WPs or caseins, through thiol–disulphide interchanges (Fenelon, Hickey, Buggy, McCarthy, & Murphy, 2018). Conformational changes of WPs may have technological and functional consequences. When denatured WPs form aggregates and associate to the casein micelles, voluminosity increases leading to an increase in viscosity (Anema, Lowe, Lee, & Klostermeyer, 2014; Anema, Lowe, & Li, 2004; Ho et al., 2019). High viscosities are undesirable during processing, creating fouling on the heat exchangers, reducing spray-drying efficiency and affecting solubility and bulk density of the resulting powder (Petit, Six, Moreau, Ronse, & Delaplace, 2013; Schuck et al., 2005). Moreover, the

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extent of denaturation of WP has been shown to impact emulsion formation and stability, which are both relevant during IMF manufacture (Dapueto, Troncoso, Mella, & Zúñiga, 2019).

The presence of other components, such as lactose and calcium, protein concentration and dry matter level affect conformational changes of WP. Anema, Siew, and Klostermeyer (2006) demonstrated that increasing concentration of lactose or non-protein soluble components retarded β-lactoglobulin and α-lactalbumin irreversible denaturation, while the level of denaturation of both proteins increased as the protein concentration in milk increased. Furthermore, Anema (2000) reported that increasing milk solids concentration reduced β-lactoglobulin denaturation, probably due to the increase in lactose concentration.

Previous studies have demonstrated the chaperone activity of caseins, stabilising other milk and non-milk proteins, by preventing their aggregation and solubility loss (Kehoe & Foegeding, 2011; Matsudomi, Kanda, & Moriwaki, 2004; Morgan, Treweek, Lindner, Price, & Carver, 2005; O'Kennedy & Mounsey, 2006; Yong & Foegeding, 2010). Furthermore, interaction between inulin and WP has also been studied. Tobin et al. (2010) produced micro-particulated WP concentrates and found that partially replacing lactose by inulin increased WP denaturation and aggregation, and concluded that inulin does not protect WP from heat-induced denaturation as lactose. Guo, Wang, and Wang (2018) demonstrated that WP and inulin interacted impacting the gelation properties of WP. Gao et al. (2019) also reported interaction between WP and inulin during wet-heating. When heating WP in presence of inulin, the authors found an increase of the surface hydrophobicity as well as a decrease in the free-sulfhydryl group content, compared with WP in absence of inulin, indicating that inulin affects the conformation of WP and enhances the degree of aggregation. However, to the best of the authors' knowledge the interaction of casein and inulin with WP, at the concentrations found during IMF production, has not been studied.

The compositional differences between cow milk and IMF, and even the presence of components in the latter that are absent in cow milk, such as oligosaccharides, highlights the importance of studying the effect that each component exerts on the conformational changes of WP, at the levels commonly found in IMF. Thus, this study was conducted with the aim to understand how the presence of casein and inulin, at ratios relevant to IMF processing, affect the heat-induced conformational changes of WP.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI, Provon® 292, Glanbia Nutritionals Inc, Flitchburg, USA) and calcium caseinate (Lactoprot, Kaltenkirchen, Germany) were obtained from L&G S.A. (Montevideo, Uruguay). The protein content determined by Kjeldahl (total nitrogen × 6.38) was 86.99% (w/w, wet basis) in WPI and 88.39% (w/w, wet basis) in calcium caseinate. Inulin (Orafiti® GR, Beneo, Tienen, Belgium), with an average degree of polymerisation ≥10, was kindly provided by L&G S.A. (Montevideo, Uruguay).

2.2. Model systems preparation

Model systems were prepared by mixing WPI, calcium caseinate and inulin in the proportion required for infant formulae. The concentration of individual components was calculated based on a 60:40 WP-to-casein ratio in a 25% (w/w) total solids wet-mix of a formula containing whey proteins, casein, lactose, oil and oligosaccharides. The level of inulin corresponds to the maximum concentration of total oligosaccharides allowed by the European Commission (2016) in infant formulae.

Table 1 presents casein, WP and inulin content of the model systems. The required quantities of each ingredient were slowly added to distilled water at room temperature (approximately 25 °C) during stirring. The order of addition of the ingredients was: WPI, calcium caseinate and/or inulin. The systems were further stirred for 1 h at room temperature, divided into four 40 mL-subsamples and subjected to the corresponding heat treatment (unheated, 66, 70 or 75 °C).

The heat treatments were performed at the desired temperature for 30 min in a temperature-controlled water bath with stirring (JP SELECTA, Barcelona, Spain). The mildest heat treatment (66 °C−30 min) was chosen as it corresponds to the batch pasteurisation conditions for dairy products with a total solids level higher than 18% (FDA, 2017). Heat treatments at 70 and 75 °C were also included in the study to investigate the impact of applying more severe heat treatments. The systems were then rapidly cooled down in ice baths to prevent further heating. All the treated systems were stored at 2 °C overnight before being freeze-dried (VirTis BenchTop K, SP Industries, Warminister, PA, USA) for further analyses. The freeze-dried systems were used for all the analyses except the protein solubility that was measured in the liquid systems before freeze-drying.

2.3. Particle size distribution

The freeze-dried model systems were dispersed in simulated milk ultrafiltrate (SMUF, Jenness & Koops, 1962) at 1% (w/v) at room temperature (approximately 25 °C) and stirred for 1 h. Intensity-weighted size distributions was measured at 25 °C after 60 s of equilibration in a glass cell, using dynamic light-scattering (NanoPlus 3, Particulate Systems, Norcross, GA, USA). A solvent viscosity of 0.89 mPa s and refractive index of 1.33 were used for the calculations. Each system was analysed in triplicate and three measurements with an accumulation time of 50 were taken for each replicate.

2.4. Protein profile of the unheated and heated model systems under reducing and non-reducing conditions

SDS−PAGE was performed under reducing and non-reducing conditions using Mini Protean II Dual Slab Cell (BIO-RAD, Hercules, CA, USA). Continuous and stacking gels of 12 and 4% acrylamide, respectively, were prepared. A running buffer system pH 8.3 containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS was used. The freeze-dried model systems were reconstituted in a sample buffer system (6% SDS, 30% glycerol and 0.03% bromophenol blue) with or without β-mercaptoethanol. Coomassie brilliant blue R250 was used as the colorant agent. Low molecular mass markers (GE Healthcare 17044061, Chicago, IL, USA) included phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa),

Table 1
Whey protein, casein and inulin content in the model systems.[a]

Model system	Whey protein (%, w/w)	Casein protein (%, w/w)	Inulin (%, w/w)
WP	1.8	0.0	0.0
WP-CAS	1.8	1.2	0.0
WP-INUL	1.8	0.0	1.5
WP−CAS-INUL	1.8	1.2	1.5

[a] Whey protein is that in added WPI; casein protein is that in added calcium caseinate.

carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and lysozyme (14 kDa).

2.5. Infrared spectroscopic measurements and data processing

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR/FT-IR) spectra were recorded with a Thermo Nicolet iS10 FTIR spectrophotometer (Thermo Scientific, Waltham, MA, USA) in the 4000 to 650 cm^{-1} range at room temperature with a spectral resolution of 4 cm^{-1}. To improve the signal-to-noise ratio, 164 scans were acquired for each spectrum. Two technical replicates and three biological replicates of the freeze-dried systems were averaged getting one average spectrum for each sample. Second derivatives were calculated on the averaged spectra using the Savitzky–Golay algorithm with 9-point smoothing. This procedure was carried out to increase the number of discriminative features inside the broad spectral bands, to help in the band assignment, and to minimise problems with baseline shifts (Naumann, 2000).

The chemical composition and protein conformation study was carried out analysing amide I (1700–1600 cm^{-1}), amide II (1600–1480 cm^{-1}) and amide III regions (1320–1220 cm^{-1}). The general band assignment in our average second derivative spectra, was performed taking into account the band assignment reported in the literature, generally obtained from known globular proteins (Fabian & Mäntele, 2006; Ferrer, Bosch, Yantorno, & Baran, 2008; Grewal, Huppertz, & Vasiljevic, 2018; Rahaman, Vasiljevic, & Ramchandran, 2015). This resulted in identifying five mayor bands as following: α-helix at 1652 cm^{-1} (1656–1646 cm^{-1}), β-turns at 1674 cm^{-1} (1679–1667 cm^{-1}), random coil at 1645 cm^{-1} (1646–1639 cm^{-1}), and β-sheets regions that includes the high energy contribution in the 1697–1675 cm^{-1} region, associated to intermolecular β-sheet with peaks at 1693 and 1688 cm^{-1}, and the low energy β-sheets contributions in the 1639–1610 cm^{-1} region, which comprises the intramolecular β-sheet peak at 1636 cm^{-1} and the intermolecular anti-parallel β-sheet region (1621–1610 cm^{-1}) with peaks at 1628, 1624 and 1617 cm^{-1} (Bogahawaththa, Chandrapala, & Vasiljevic, 2019; Grewal, Chandrapala, Donkor, Apostolopoulos, & Vasiljevic, 2017; Grewal et al., 2018; Mediwaththe, Bogahawaththa, Grewal, Chandrapala, & Vasiljevic, 2018; Rahaman et al., 2015).

Different cluster analyses were carried out to determine the heterogeneity and/or variability among the FT-IR/ATR average spectra. For this purpose, the second-derivatives of the 16 average spectra vector-normalised in the whole range (4000–650 cm^{-1}) were used as input data. The spectral distance values were calculated at different wavenumber ranges as indicated in each case using the "scaling to first range" method. Clustering was carried out using Ward's algorithm. Data preprocessing and data analysis were carried out with the OPUS software (versions 4.2; and 7.0 Bruker Optics GmbH, Ettlingen, Germany).

2.6. Temperature and degree of denaturation of whey proteins

Temperature of denaturation (Td) and degree of denaturation were determined using a differential scanning calorimeter (Q2000 DSC, TA Instruments, New Castle, DE, USA) calibrated with Indium. The freeze-dried model systems were rehydrated in distilled water to ~30% (w/w) total solids. Samples of 15–20 mg of the resuspended systems were weighed into aluminium pans and hermetically sealed. An empty pan was used as reference. Samples were equilibrated at 20 °C for 3 min, then heated until 130 °C at 10 °C min^{-1} and finally equilibrated at 130 °C for 3 min. After scanning, the pans were perforated and dried in an oven at 102 °C for 16 h, to determine the total solids content in each pan. Experiments were performed in triplicates. The peak minimum of the endotherm was defined as denaturation temperature (Td). The degree of denaturation was calculated according to Eq. (1).

$$\text{Degree of denaturation}(\%) = 100 - \frac{\Delta H_i}{\Delta H_{UH}} \times 100 \quad (1)$$

where ΔH_i is the area of the endotherm (expressed in J g^{-1} WP) of the heated system and ΔH_{UH} is the area of the endotherm (expressed in J g^{-1} WP) of the corresponding unheated system.

2.7. Protein solubility

For the soluble protein quantification, 1 mL of each liquid system was centrifuged (Sigma 6-16KS, Osterode am Harz, Germany) at 20,000 × *g* for 20 min at room temperature (approximately 25 °C). The protein content in the supernatant (soluble protein content) was determined by Bradford (1976), using a calibration curve of WPI ($y = 0.0198x$, $r^2 = 0.9494$), calcium caseinate ($y = 0.026x$, $r^2 = 0.9964$) or WPI:calcium caseinate 1.5:1 ($y = 0.0262x$, $r^2 = 0.9687$), depending on the system composition. The absorbance was measured at 595 nm (Mettler Toledo UV5, Columbus, OH, USA) after 10 min. The protein solubility was determined in triplicates and calculated using Eq. (2).

$$\text{Protein solubility}(\%) = \frac{\text{Soluble protein content}}{\text{Total protein content}} \times 100 \quad (2)$$

where total protein content (%, w/w) is the protein concentration in the model systems as determined by Kjeldahl (total nitrogen × 6.38) and the soluble protein content (%, w/w) is the protein concentration in the supernatant as determined by Bradford.

2.8. Statistical analysis

JMP® 12.1.0 software (SAS Institute Inc., North Carolina, USA) was used for analysis of variance (ANOVA) and Tukey's multi-comparison test. The level of significance was determined at $P < 0.05$.

3. Results

3.1. Particle size distribution

The unheated model system containing only WPI (WP system) had a mean particle size of 238 ± 39 nm (Fig. 1), in agreement with previous studies (Gao et al., 2019). When caseinate was added (WP–CAS system) the particle size distribution (PSD) was bimodal. The first population, which ranged between 19 and 28 nm, probably corresponds to disaggregated caseins. The second population had a mean particle size of 323 ± 20 nm, therefore, the presence of calcium caseinate increased ($P < 0.05$) the mean particle size of WP system. Addition of inulin to WP did not change the PSD of the unheated system nor increased the mean particle size ($P > 0.05$). Addition of both caseinate and inulin (WP–CAS–INUL system) resulted in a bimodal PSD similar to the one obtained for the WP–CAS system. The second population of WP–CAS–INUL unheated system was in average (312 ± 26 nm) significantly greater ($P < 0.05$) than unheated WP, but similar ($P > 0.05$) to WP–CAS, suggesting that the PSD was mainly affected by the presence of calcium caseinate.

Heat treatment of WP system at 66 or 70 °C did not modify the PSD. However, a significant increase ($P < 0.0001$) in the mean diameter was obtained after the heat treatment at 75 °C

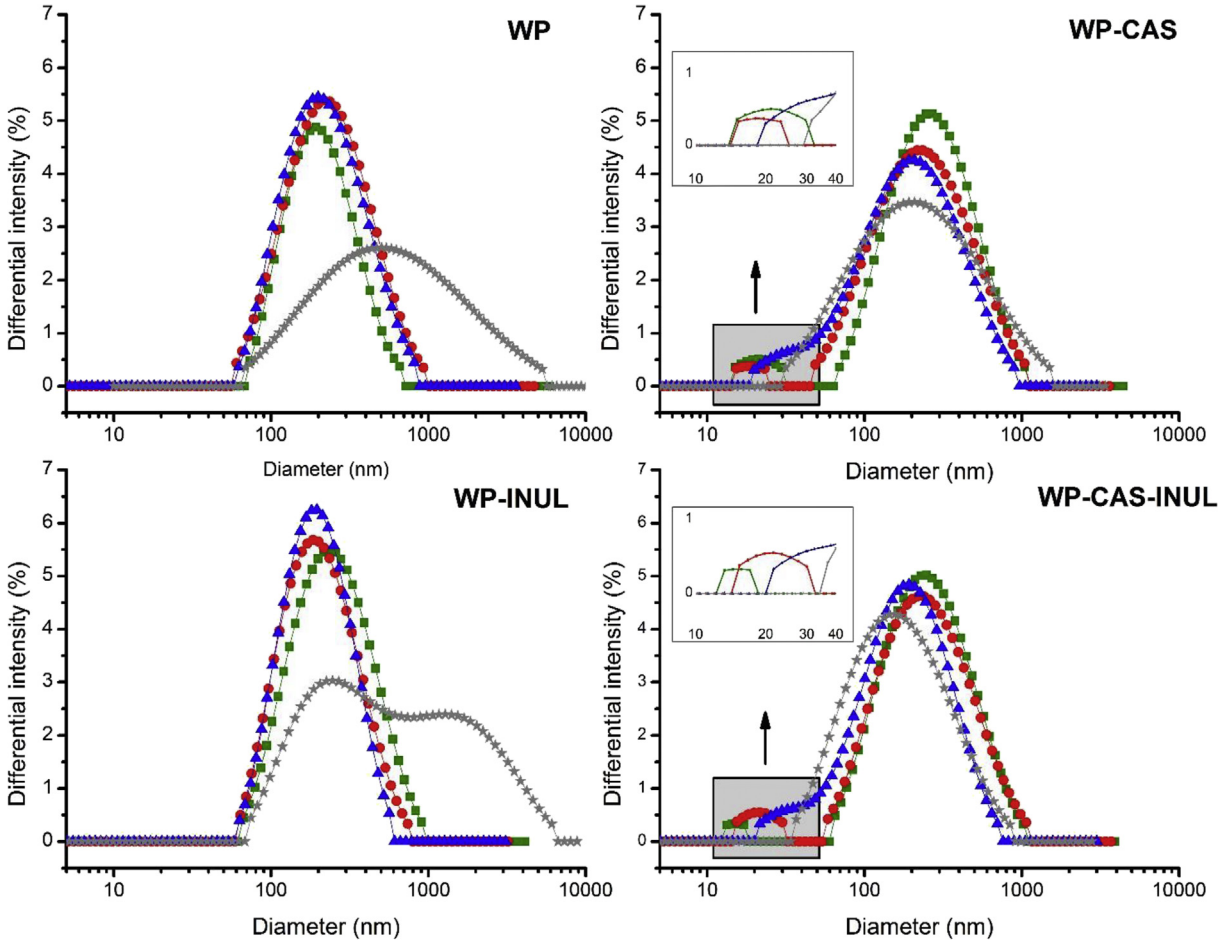

Fig. 1. Particle size distribution of the WP, WP–CAS, WP–INUL and WP–CAS–INUL model systems, unheated (■) and heated at (●) 66, (▲) and (★) 75 °C for 30 min.

(932 ± 57 nm), indicating formation of aggregates (Fig. 1). The PSD of WP–CAS was also not modified by heat treatments at 66 and 70 °C. At 75 °C, the presence of small size a population was not detected, suggesting aggregation of individual caseins (Fig. 1). The main peak of WP–CAS system widened with heat treatments; however, the mean particle size did not increase with temperature ($P > 0.05$). The WP–INUL system behaved similarly to the WP system in that the PSD did not show presence of large aggregates until heat treatment was performed at 75 °C. However, after heating at 75 °C a bimodal distribution was obtained, with a first population averaging 312 ± 14 nm and a second population averaging 2048 ± 107 nm. Therefore, aggregates were formed in the presence of inulin after 30 min at 75 °C showing a bimodal PSD. On the other hand, WP–CAS–INUL had a behaviour similar to that of WP–CAS, with individual caseins not associated to the micelle until after the 75 °C heat treatment and the absence of large aggregates (Fig. 1).

3.2. Protein profile of the unheated and heated model systems under reducing and non-reducing conditions

Fig. 2 shows SDS–PAGE of unheated and heated model systems. Under non-reducing conditions, as the intensity of heat treatment increases, a reduction in the bands corresponding to minor WP, such as lactoferrin (80 kDa) and heavy chain immunoglobulin is observed (50–70 kDa), while aggregates of high molecular mass appeared on the top of the gel (Fig. 2A) (Butler, 1969; Lönnerdal & Suzuki, 2013; Triani & Foegeding, 2019). The formation of large aggregates on the top part of the gel after heat treatment of WP has been previously reported (Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013; Markoska, Huppertz, Grewal, & Vasiljevic, 2019; Triani & Foegeding, 2019). On the other hand, under reducing conditions, protein profiles of WP system heated and unheated showed no differences (Fig. 2B). These results indicate that as temperature increases, aggregates of high molecular mass involving reducing bonds, such as disulphide, are formed. Gels obtained for WP–INUL system were similar to those obtained for WP, indicating that inulin did not affect significantly the formation of WP aggregates mediated by disulphide bonds. However, WP–CAS system gels obtained under reducing and non-reducing conditions did not show differences in the protein profiles suggesting that caseins may affect the disulphide bonds formation between WP (Fig. 2). Finally, WP–CAS–INUL system behaved similarly to WP–CAS system.

3.3. Chemical and protein conformation analysis by infrared spectroscopy

FT-IR provides information on the overall biochemical and structural composition of the material under study. Fig. 3A depicts the average spectra obtained for each unheated model system (WP, WP–CAS, WP–INUL and WP CAS–INUL) at the mid IR spectral windows 1700–700 cm^{-1}. The spectral features of the average spectra of the unheated systems shows amide I (1700–1600 cm^{-1})

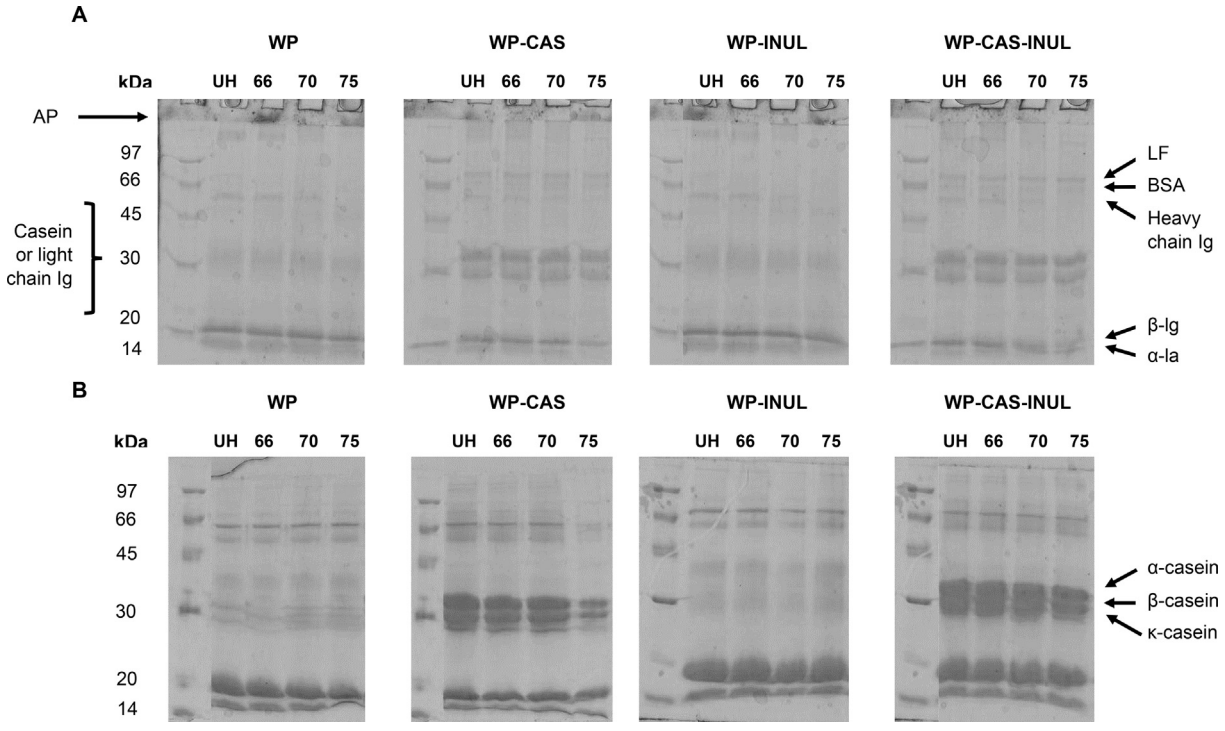

Fig. 2. Non-reducing (A) and reducing (B) SDS–PAGE analysis of the WP, WP–CAS, WP–INUL and WP–CAS–INUL model systems unheated (UH) and heated at 66, 70 and 75 °C for 30 min: α-la, α-lactalbumin; β-lg, β-lactoglobulin; LF, lactoferrin; BSA, bovine serum albumin; AP, aggregated proteins; Ig, immunoglobulin.

and amide II (1600–1480 cm^{-1}) bands as the two more intense absorption bands for WP and WP–CAS systems. The amide I band represents primarily the C=O stretching of the amide group and is directly related to protein backbone conformation while amide II band results from the N–H bending vibration and from the C–N stretching vibrations in protein and peptides. The amide III band (1320–1220 cm^{-1}), assigned to C–N stretching and N–H bending, and also conformationally sensitive (Fabian & Mäntele, 2006), though with less intensity, is present in all spectra. Finally, the "carbohydrates region" (1200–900 cm^{-1}) attributed to C–OH,

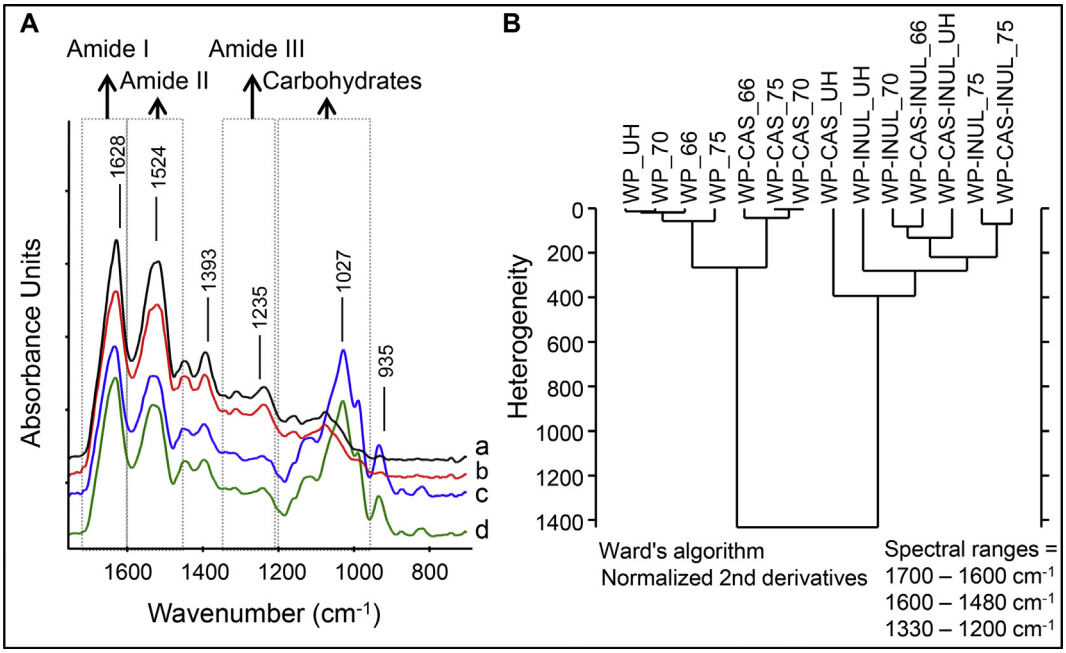

Fig. 3. Panel A: ATR/FT-IR average spectra of the WP (a), WP–CAS (b), WP–INUL (c) and WP–CAS–INUL (d) unheated model systems in the spectral region 1700–650 cm^{-1}. Spectral regions associated to functional groups in biomolecules and conformational vibrations in proteins are indicated: amide I (1700–1600 cm^{-1}), amide II (1600–1480 cm^{-1}), amide III (1320–1220 cm^{-1}), and "carbohydrate region" (1200–900 cm^{-1}). Panel B: cluster analysis obtained with 2nd derivatives of the average spectrum of each system (16 average spectra) vector normalised in the whole range (4000–640 cm^{-1}) as input data. Scaling to first range was applied for spectral distance calculation using the three amide regions (amide I, II and III). The dendrogram was constructed using Ward's algorithm.

C–O–C and C–O vibrations in polysaccharides (Naumann, 2000) arises quite prominent in WP samples containing inulin due to the absorbance of polysaccharides of inulin (Wang, Wang, Sun, & Sun, 2019).

As the original spectra did not reveal sufficient chemical and/or structural information, the number of discriminative features inside the broad bands was enhanced by the calculation of the second derivatives of the average spectra (Fabian & Mäntele, 2006; Naumann, 2000). The spectral features of these second derivatives vector normalised spectra were first subjected to a multivariate cluster analysis in the amide regions sensitive to protein conformation (amide I, II and III; Fig. 3B). The chemical composition and conformational changes that casein and inulin produced in WP under heat treatment were analysed. The dendrogram obtained separated the average spectra of the heated an unheated systems into two main clusters mainly dominated by the chemical composition. It is interesting to remark the significant chemical and structural changes that casein and inulin produced on WP. All the WP average spectra, unheated and heated even up to 75 °C, were grouped together in a cluster separated from the other systems. Furthermore, the biochemical effect produced by inulin in WP, alone or together with casein, were much more significant than the effects produced by casein alone. This could be explained by protein-carbohydrate interactions, which result in the attachment of inulin to WP (Wang, Bao, & Chen, 2013). Therefore, the significant spectral differences observed at amide regions between the systems with inulin (WP–INUL and WP–CAS–INUL) and without inulin (WP and WP–CAS) (Fig. 3A), are confirming the impact in both chemical and secondary structure produced by WP interaction with inulin (Jia et al., 2016).

To carry out a more in-depth analysis of the effect of casein and inulin in WP system conformation at different temperatures, we further studied the spectral features of the second derivatives vector normalised spectra in amide I region (Fig. 4A). The amide I has largely been selected because of its high sensitivity to small variations in the molecular geometry and hydrogen bonding patterns originated mainly from C=O stretching frequency. Changes in the absorption position of peaks and/or their intensities were noticed when casein, inulin, and both were included in WP systems. However, the most remarkable effects were observed in β-sheet components, which suffered shifts, rearranges and increases or decreases in their intensities. Particularly, those changes were observed in peaks at 1617, 1624, 1628 cm^{-1}, (assigned to intermolecular β-sheet), and 1688 and 1693 cm^{-1} (assigned to intermolecular β-sheet aggregation) (Grewal et al., 2018, 2017; Mediwaththe et al., 2018; Rahaman et al., 2015).

A cluster analyses was therefore performed to evaluate the effect on aggregation in WP systems in presence of inulin and casein at different heating temperatures (Fig. 4B). For this purpose the spectral region associated to high energy β-sheet conformation (1700–1685 cm^{-1}) which included peaks at 1694 and 1688 cm^{-1}, and the low energy region due to anti-parallel β-sheet (1631–1610 cm^{-1}) which included the 1628, 1624 and 1617 cm^{-1} peaks, was carried out. The most notable result was that WP sample heated at 75 °C clustered alone separated at a high spectral distance from the unheated and also heated at 66 and 70 °C WP samples. This spectral dissimilarity put in evidence the significant changes in aggregation that occurred when WP system is subjected to high temperature treatment (75 °C) without casein or inulin. Interestingly, WP–CAS average spectrum also clustered alone, quite separated from all WP–CAS–INUL systems. This result might be due to the contribution of casein aggregates of different sizes, which as mentioned in section 3.1, are present in the WP–CAS systems. This result is in agreement with a recent FT-IR casein characterisation study that addressed the issue that caseins behave differently from other proteins because of the interaction of amino acids which results in a protein conformation that resembles a denatured globular protein (Siroëić, Krehula, Katanëić, & Hrnjak-Murgić, 2016).

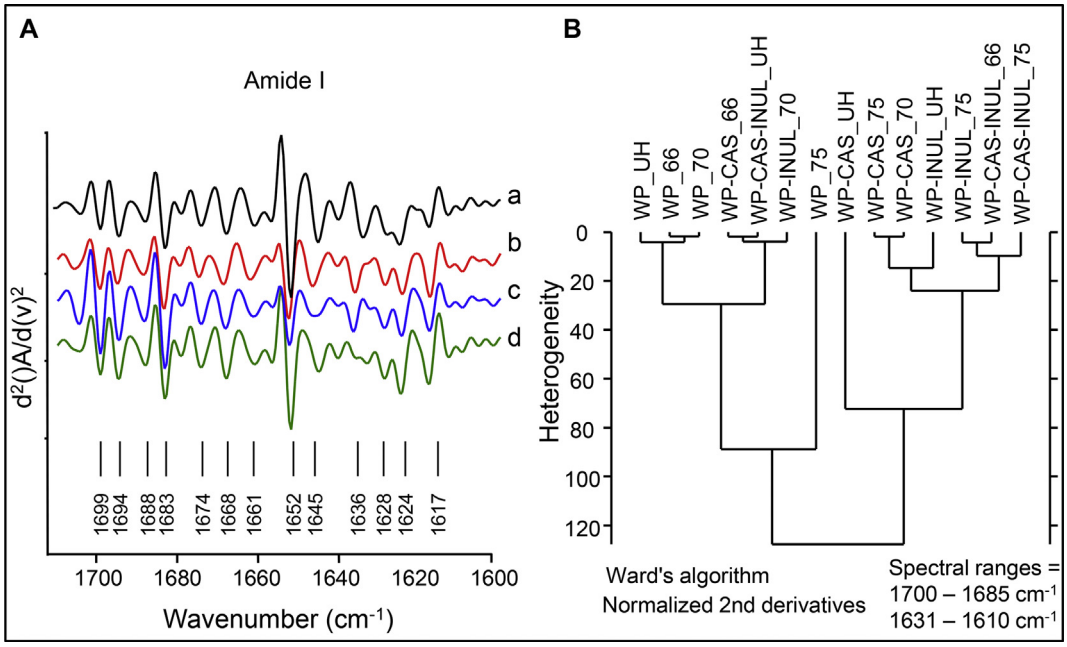

Fig. 4. Panel A: vector-normalised 2nd derivatives of the average spectrum of the WP (a), WP–CAS (b), WP–INUL (c) and WP–CAS–INUL (d) unheated model systems in the amide I region (1700–1600 cm^{-1}). Panel B: cluster analysis obtained with the 2nd derivatives of the average spectrum of each system vector normalised (16 average spectra) as input data, using scaling to first range for spectral distance calculation in the spectral ranges associated to protein aggregation: high energy contribution of β-sheets aggregates (1700–1675 cm^{-1}) and low energy intermolecular β-sheets contributions (1621–1610 cm^{-1}). The dendrogram was constructed using Ward's algorithm.

3.4. Temperature and degree of denaturation of whey proteins

DSC analyses were carried out to determine the degree of denaturation of WP after heat treatment as well as the temperature of denaturation of the different model systems. The shape of the thermograms (Fig. 5) was similar to those reported in the literature (Fitzsimons, Mulvihill, & Morris, 2007; Murphy, Fenelon, Roos, & Hogan, 2014). The thermogram of the unheated WP system presented a minimum (Td) at 74.6 ± 0.2 °C (Table 2), which is coincident with the Td of β-lactoglobulin (Boye & Alli, 2000). Td of α-lactalbumin was identified as a shoulder at T~ 65 °C (Fig. 5), and was noticeable only in the WP system (Boye & Alli, 2000). α-Lactalbumin has lower Td than β-lactoglobulin and therefore the shoulder disappears faster with heat treatment than the endormethic peak associated with β-lactoglobulin denaturation (Fig. 5).

The effect of the addition of casein or inulin on the denaturation of WP was also evidenced by the ΔH of the unheated systems being 10.60 ± 0.41 J g^{-1} WP for WP system; 7.89 ± 0.66 J g^{-1} WP for WP–CAS system, 7.20 ± 0.73 J g^{-1} WP for WP–INUL system and 7.99 ± 0.34 J g^{-1} WP for WP–CAS–INUL system. These results indicated that the WP were modified by the presence of inulin or casein in the model systems. The decrease of WP–CAS and WP–INUL denaturation enthalpies (expressed in g of WP) compared with the denaturation enthalpy of WP system; indicates that both induce changes in WP that may affect thermal denaturation.

Furthermore, the addition of caseinate did not affect Td of WP, while inulin significantly increased ($P < 0.05$) Td of the unheated systems. Heat treatment did not exert any significant effect ($P > 0.05$) on Td of the WP system, until 75 °C when an increment in Td was observed (Table 2). Similar results were obtained for WP–INUL, while Td of WP–CAS and WP–CAS–INUL systems significantly increased ($P < 0.05$) after heat treatments at 70 and 75 °C (Table 2).

The degree of denaturation of WP system increased significantly ($P < 0.05$) with every heat treatment, although the highest increase in denaturation was obtained after heating at 75 °C, when WP system was 79.9 ± 0.6% denatured (Fig. 6). WP–CAS system had a significantly higher degree of denaturation than WP system ($P < 0.05$) after heat treatments at 70 °C and 75 °C, and ended up with 93.0 ± 1.8% WP denaturation after 30 min at 75 °C (Fig. 6). When inulin was added alone to WP, denaturation increased after heat treatment at 66 °C ($P < 0.05$), did not change after heating at 70 °C ($P < 0.05$) and increased again ($P < 0.05$) after a 75 °C heat treatment. In presence of both casein and inulin, denaturation increased with every heat treatment. WP–CAS–INUL system showed the highest level of denaturation after every heat treatment, except for 66 °C, when denaturation in WP–INUL was significantly higher ($P < 0.05$). WP–CAS–INUL system heated at 75 °C had the highest level of WP denaturation, followed by WP–CAS, WP–INUL and WP (Fig. 6).

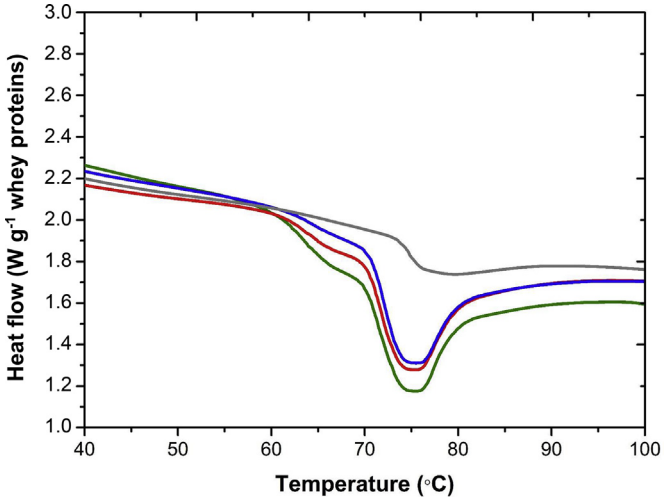

Fig. 5. Thermograms obtained for the WP model system unheated (—) and heated at (—) 66, (—) 70 and (—) 75 °C for 30 min.

Table 2
Denaturation temperatures (°C) obtained for the model systems unheated and heated at 66, 70 and 75 °C for 30 min.[a]

Model system	Unheated	Heated		
		66 °C	70 °C	75 °C
WP	74.6 ± 0.2^{aA}	74.6 ± 0.1^{aA}	74.7 ± 0.2^{aA}	77.6 ± 0.2^{bA}
WP-CAS	74.8 ± 0.3^{aAB}	74.6 ± 0.3^{aA}	77.5 ± 0.4^{bC}	80.1 ± 0.7^{cBC}
WP-INUL	75.7 ± 0.4^{aC}	75.6 ± 0.0^{aB}	75.4 ± 0.3^{aB}	79.3 ± 0.1^{bAB}
WP–CAS-INUL	75.4 ± 0.2^{aBC}	76.0 ± 0.3^{aB}	78.8 ± 0.1^{bD}	81.5 ± 1.1^{cC}

[a] Values are means ± standard deviation (n = 3); different lower case and upper case superscript letters indicate significant differences (Tukey test, $P < 0.05$) among heat treatments and among systems, respectively.

3.5. Protein solubility

Protein solubility of the unheated model systems varied between 98.6 ± 1.9% for WP and 87.4 ± 0.7% for WP–CAS–INUL (Fig. 7). Before heat treatment, addition of caseinate resulted in a reduction of protein solubility ($P < 0.05$), while addition of inulin alone did not affect solubility (WP = WP–INUL). WP–CAS–INUL unheated system had a lower ($P < 0.05$) protein solubility than WP and WP–INUL and similar to WP–CAS ($P > 0.05$, Fig. 7).

Heating WP model system at 66 and 70 °C did not affect protein solubility ($P > 0.05$), while heat treatment at 75 °C significantly reduced solubility ($P < 0.0001$) by 25% (Fig. 7). In the WP–CAS system, solubility did not undergo any significant change with heat treatment ($P > 0.05$) until 75 °C, when it increased significantly ($P = 0.0005$) reaching the highest solubility (101.4 ± 3.1%). On the other hand, solubility of WP–INUL system decreased with temperature, from 95.3 ± 0.9% (unheated) to 64.7 ± 0.3% (heated at 75 °C). Finally, protein solubility in WP–CAS–INUL was constant with heat treatment.

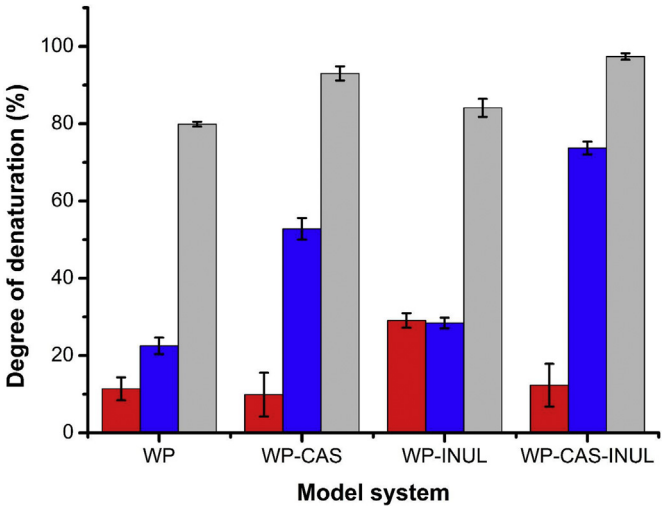

Fig. 6. Degree of denaturation of the WP, WP–CAS, WP–INUL and WP–CAS–INUL model systems heated at (■) 66, (■) 70 and (■) 75 °C for 30 min; bars represent standard deviation (n = 3).

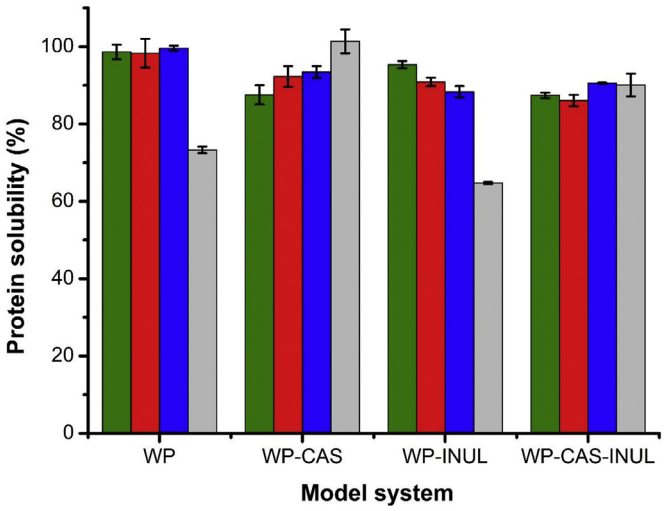

Fig. 7. Protein solubility of the WP, WP–CAS, WP–INUL and WP–CAS–INUL model systems, unheated (■) and heated at (■) 66, (■) 70 and (■) 75 °C for 30 min; bars represent standard deviation (n = 3).

To understand the effect of casein addition, a 1.2% calcium caseinate system was prepared (CAS) and heated under the same conditions. Solubility of CAS was constant with heat treatment being 100.8 ± 1.4% in average (data not shown). Hence, the increase in solubility obtained in the casein containing systems heated at 75 °C is not due to a solubilisation of caseins but to a reduction in the loss of solubility of WP.

4. Discussion

The results obtained in the present study indicate that WPs are affected by the heat treatment but also by the presence of other components in the milk formulation.

When the WP system was subjected to heat treatment in absence of other components, conformational changes were observed, that were particularly noticeable after heating at 75 °C for 30 min. β-Lactoglobulin comprises approximately 50% of the total WP and is irreversibly denatured when heated at temperatures above 70 °C. After unfolding, its reactive thiol group is exposed and aggregations via −SH/S−S interchange reactions or non-covalent reactions can take place if further heating is applied (Wijayanti, Brodkorb, Hogan, & Murphy, 2019). On the other hand, denaturation of α-lactalbumin, the second most abundant protein in whey, is reversible at temperatures below 80 °C, due to the lack of a free −SH in its structure (Wijayanti et al., 2019).

The level of WP denaturation increased with the temperature of the heat treatment, ending with almost 80% denaturation after a 75 °C heat treatment (Fig. 6). Unfolding and exposing of free thiol groups lead to aggregation, as evidenced by dynamic light-scattering, SDS–PAGE and FT-IR analysis of the spectral region associated to β-sheet conformation. SDS–PAGE confirmed that the aggregates were associated by reducing bonds (Fig. 2). Aggregates formed during heat treatment at 75 °C had large particle size (Fig. 1), which in turn provoked a significant ($P < 0.05$) reduction in the protein solubility (Fig. 7). Protein solubility of WP, that was 98.6% in the unheated system, remained stable during the 66 and 70 °C heat treatments. However, and in line with particle size results, after heating at 75 °C for 30 min, protein solubility decreased by 25%.

The effect of casein presence was studied in the unheated and heated systems. In the unheated systems, addition of casein reduced protein solubility. This result could be explained by the formation of WP–WP or WP–casein aggregates or by the reduction in the available water in the system. Adding caseinate did not have a significant effect on Td (Table 2) of the unheated systems. Similarly, addition of β-casein did not alter Td of β-lactoglobulin, as reported by Kehoe and Foegeding (2011). Upon heating, the presence of casein affected the behaviour of WP. The PSD results showed that, after heating at 75 °C, systems containing casein (WP–CAS and WP–CAS–INUL) did not present large aggregates, as observed in the model systems without casein (Fig. 1). This result is in agreement with previous studies showing that aggregates formed in presence of caseins are of smaller size than the ones formed in absence of caseins (Gaspard, Auty, Kelly, & Mahony, 2017; Guyomarc'h, Nono, Nicolai, & Durand, 2009; Kehoe & Foegeding, 2011; Mounsey & O'Kennedy, 2009). Moreover, the SDS–PAGE results showed that no aggregation between WP through reducing bonds was obtained in the systems containing casein, unlike the observed for the WP and WP–INUL systems (Fig. 2).

The chaperone activity of caseins, which protect WP against aggregation, has been described in the literature, although the mechanism is not yet fully understood. Gaspard et al. (2017) reported that κ-casein stabilised WP aggregates during heating and that the presence of α_S- and β-casein enhanced the stability. Kehoe and Foegeding (2011) studied the chaperone-activity of β-casein and proposed that β-casein competes with WP, forming aggregates of smaller size than the ones formed during WP self-aggregation. As a result of the formation of aggregates of smaller size, the protein solubility increases. The effect on solubility was also verified in the current study (Fig. 7). While solubility of WP system decreased significantly ($P < 0.0001$) after 30 min at 75 °C, solubility of WP–CAS increased significantly by 8.5% after the same heat treatment. Consequently, the solubility results supported the hypothesis of the chaperone-like activity of caseins. The inhibition of the formation of large heat-induced aggregates of WP has been verified for calcium caseinate in the present work, and for different casein sources, such as sodium caseinate, milk protein concentrate, and κ-, β- and α_{S1}-caseins in previous studies (Gaspard et al., 2017; Guyomarc'h et al., 2009; Kehoe & Foegeding, 2011; O'Kennedy & Mounsey, 2006; Singh, Prakash, Bhandari, & Bansal, 2019). The stabilisation of β- and α_{S1}-caseins has been explained by preferred hydrophobic interactions between the caseins and the unfolded WP, while in the chaperon activity of κ-casein disulphide bridges may also take part, given the presence of cysteine residues in κ-casein (Guyomarc'h et al., 2009).

In contrast, the addition of casein increased the level of denaturation of the systems treated at 70 and 75 °C (WP–CAS > WP). Calcium caseinate was used as casein source; therefore it might be possible that free calcium was added to the system, which has been shown to increase WP denaturation. Joyce, Kelly, and O'Mahony (2018) reported that the addition of 2.2 mM Ca increased WP denaturation and aggregation. While the increase on denaturation was verified by our results, such effect was not observed on the aggregation. It can be hypothesised that the presence of casein, preventing the WP aggregation, prevailed over the role of calcium as promoter of aggregation.

The effect of the presence of inulin was also studied. According to the PSD results, the addition of inulin did not increase the particle size of the unheated systems (Fig. 1), although a reduction in the enthalpy was obtained, suggesting interaction between WP and inulin. Similar findings were reported by Gao et al. (2019), who suggested that WP at their native state and inulin interact through hydrogen bonding. Moreover, the presence of inulin significantly increased ($P < 0.05$) the Td of the unheated systems (Table 2), indicating that INUL may interact with WP modifying protein conformation as was observed in FT-IR cluster of β-sheet

conformation region (Fig. 4B). Upon heating at 75 °C, inulin did not increase the degree of denaturation (Fig. 6) or aggregation of the WP. As shown in Fig. 1, after heating at 75 °C a bimodal PSD with a population of large particle size appeared in the system (indicating formation of aggregates), although the distribution was different to the one observed for the system WP (monomodal). Similarly, Guo et al. (2018) obtained multimodal PSD for WPI (4–8%) + inulin (1–5%) mixtures heated at 85 °C for 30 min. Furthermore, addition of inulin to WP and WP–CAS systems resulted in lower protein solubility (Fig. 7). The lower solubility obtained for WP–INUL as compared with WP, when heated at 75 °C, could be explained by the different PSD (Fig. 1).

In the presence of both casein and inulin, the effect of casein preventing large aggregates formation and loss of solubility after a 75 °C heat treatment was still evident (Figs. 1 and 7).

5. Conclusions

The present study demonstrated that heat induced denaturation, aggregation and loss of solubility of WP, are modified by the presence of inulin or casein.

Inulin induced conformational changes in WP, as determined by DSC and FT-IR. Furthermore, for the unheated systems, adding inulin to WP reduced solubility by 10%.

Denaturation of WP as determined by DSC, occurred during heating in presence of caseins. However, caseins exerted a chaperone activity, preventing WP aggregation and loss of solubility.

This study provided relevant information to the infant formula production, unravelling the individual effect of casein and inulin on the heat induced conformational changes of WP.

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