Food Chemistry 115 (2009) 1479–1485

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Whey protein aggregate formation during heating in the presence of κ-carrageenan

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ARTICLE INFO

Article history:
Received 10 August 2008
Received in revised form 24 December 2008
Accepted 27 January 2009

Keywords:
Whey protein aggregation
κ-Carrageenan
SE-HPLC
MALLS
Light scattering

ABSTRACT

The effect of a negatively charged polymer, κ-carrageenan, on the aggregation behaviour of whey proteins during heating was studied. Aqueous solutions of whey protein isolate (WPI) at 0.5% were heated in the presence of κ-carrageenan (0.1%) at pH 7.0. This concentration was chosen as optimal in the detection of the intermediate aggregates during chromatographic analysis. The residual unaggregated protein, the intermediate aggregates and the soluble aggregates were all examined as a function of heating time and temperature, using size-exclusion chromatography coupled with light scattering detection. The presence of κ-carrageenan did not affect the aggregation of whey proteins heated at 75 °C; however, a change in the mechanism of aggregation seemed to occur at higher temperatures, and intermediates with higher molecular mass formed at 85 °C. At 90 °C, in the presence of κ-carrageenan, the extent of WPI aggregation was much larger, as soluble aggregates were no longer present and less residual protein was recovered in the unaggregated peak.

1. Introduction

Whey proteins are important in the food industry, not only as components of dairy products, but also as ingredients in non-dairy food products. The most abundant whey proteins, β-lactoglobulin (β-lg) and α-lactalbumin (α-la), contribute greatly to the functional properties of whey ingredients, and have been studied in great detail. However, it is their stability after thermal processing which continues to be a challenge for the food industry. The principal component of whey proteins is β-lg, a globular protein with a molar mass of 18.3 kDa, containing two disulfide bonds and one free thiol group (Swaisgood, 1992). With heating, this protein dominates the aggregation and gelation behaviour of whey proteins (Schokker, Singh, Pinder, Norris, & Creamer, 1999). The second most abundant protein, α-la, is also globular, with a molar mass of 14.2 kDa (Swaisgood, 1982). This protein shows heat-denaturation at relatively low temperatures (about 65 °C) compared to β-lg, but does not form large aggregates or gels when heated in isolation (McGuffey, Epting, Kelly, & Foegeding, 2005). However, when heated with β-lg, the inaccessible thiol groups of α-la react with those of β-lg, and α-la is incorporated in the aggregates. Under these heating conditions, α-la oligomers are also found (Schokker, Singh, & Creamer, 2000).

The aggregation of whey proteins, as well as β-lg in isolation, has been studied extensively, and the mechanisms of aggregation are well understood. Aggregation and gelation of whey proteins occurs with heating at 70 °C and above. At these temperatures, whey proteins unfold and irreversibly aggregate through exposed hydrophobic groups (Schokker et al., 2000). At low ionic strength and neutral pH the thiol group of β-lg acts as an initiator of disulfide exchange reactions and plays a very important role in the formation of the aggregates and the gel network (Hoffmann, Sala, Olieman, & de Kruif, 1997; Pouzot, Durand, & Nicolai, 2004). It has been reported for β-lg solutions that, with increasing heating temperature, the extent of denaturation increases and denatured β-lg monomers form well-defined clusters of about 30 nm (Aymard, Durand, & Nicolai, 1996). Size-exclusion chromatography (SEC) studies of heated β-lg solutions suggest that aggregates form by addition of dimers and oligomers of β-lg to larger aggregates via disulfide bridging and non-covalent interactions (Schokker et al., 1999). At sufficiently high protein concentrations, the aggregates grow and form a gel network.

Whey protein gelation is affected by the addition of polysaccharide (Beaulieu, Corredig, Turgeon, Wicker, & Doublier, 2005; Capron, Nicolai, & Durand, 1999; Ould Eleya & Turgeon, 2000). Polysaccharides are often added to modify the texture and microstructure of whey protein gels; however, the effect of polysaccharides during heat-induced aggregation of whey proteins is not fully understood.

The polysaccharide κ-carrageenan is widely used in dairy products as a stabilizer, and several studies have investigated the mechanism of interaction between carrageenans and milk proteins (Capron et al., 1999; Roesch, Cox, Compton, Happek, & Corredig, 2004; Snoeren, Payens, Jeunink, & Both, 1975). The κ-carrageenan conformation changes from coiled state to helical state with cooling (Rochas & Landry, 1987; Rochas & Rinaudo, 1980) but the transition temperature depends on the type of ions present and the

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0308-8146/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.01.077

concentration (Rochas & Landry, 1987). At high enough concentrations, κ-carrageenan molecules form a gel network with cooling (Gotz, Zick, Hinrichs, & Weisser, 2004; Roesch et al., 2004).

At neutral pH, both whey proteins and anionic polysaccharides are negatively charged and weak attractive or segregative forces play a role in causing the formation of complexes or phase separation. It has previously been shown that, for κ-carrageenan concentrations below phase separating regimes, the heat-induced aggregation of β-lg is not affected by the polysaccharide: during heating of β-lg at 70 °C; neither are the rates of protein denaturation or the structure of the aggregates affected by κ-carrageenan (Croguennoc, Nicolai, Durand, & Clark, 2001). It has also been reported that heating of WPI (2%) solutions at pH 7.0 in the presence of κ-carrageenan does not change the loss of native protein, but causes a decrease in the average molecular weight of the soluble aggregates formed during heating (de la Fuente, Hemar, & Singh, 2004). At high enough concentrations, systems containing both β-lg and κ-carrageenan have demonstrated accelerated heat-induced aggregation and gelation, but no direct interaction has been found between the two polymers and the gel formed consists of two phases (Capron et al., 1999; Ould Eleya & Turgeon, 2000).

The objective of this work was to examine the influence of κ-carrageenan on the heat-induced aggregation of WPI. The concentration chosen was below gelling and in a compatible regime to allow the study of the effect of this negatively charged polysaccharide on the preceding stages of gel formation. Aggregate formation was measured using size-exclusion chromatography (SEC) coupled with multi-angle laser light scattering (MALLS). With this technique, it is possible to obtain an absolute measurement of mass of the aggregates, independently of their hydrodynamic radius or the type of column used (Wyatt, 1993). SEC-MALLS has been successfully employed to determine the changes in molecular mass of β-lg and WPI aggregates when heated in isolation (Hoffmann et al., 1997; Kazmierski & Corredig, 2003; Schokker et al., 1999, 2000), and in the presence of polysaccharides (Beaulieu et al., 2005; de la Fuente et al., 2004).

2. Materials and methods

Whey protein isolate (WPI) was purchased from New Zealand Dairy Products (Alacen 895, Mississagua, ON). The protein (6.38 × N) was 93.7%. κ-carrageenan, from *Eucheuma Cottonii*, was donated by Danisco Canada Inc. (Mississauga, ON). Stock solutions (1% w/v) of WPI and κ-carrageenan were prepared by adding the powder, under constant stirring, to 100 mM imidazole buffer at pH 7.0. The WPI solution was gently stirred for 15–20 min and filtered through a 0.2 μm filter before use to remove any large aggregates (Nylon Millipore, Fisher Sci. Mississagua, ON). The κ-carrageenan was also gently stirred whilst being heated to 80 °C and held for 15 min and whilst cooling to 35 °C. The mixtures and control samples were prepared to a final concentration of 0.5% WPI and 0.1% κ-carrageenan. These concentrations were found optimal to detect intermediate aggregates during the chromatographic analysis.

After mixing, control samples containing only whey protein (at 0.5%) or whey protein and κ-carrageenan (at 0.5% and 0.1%, respectively) were transferred to small capped 2 ml glass vials. The vials were then heated in a thermostatically-controlled water bath at 75, 80 or 90 °C for times ranging from 0 to 20 min. After heating, the samples were immediately cooled in ice water and held at 20 °C. Samples were then filtered through a 0.45 μm filter (Nylon, Millipore, Fisher Sci.) before injection (100 μl) into the SEC-MALLS apparatus. The chromatography system consisted of an HPLC system with degasser, autosampler and UV detector (ThermoFisher, Mississagua, ON). A 0.1 μm in-line filter (Millipore, Fisher Sci.) for the mobile phase was situated before the injector. The mobile phase, 100 mM imidazole buffer at pH 7.0, was filtered through 0.2 and 0.1 μm filters, twice before use. Samples were eluted at a flow rate of 0.5 ml/min on three columns (300 × 7.8 mm) connected in series (Biosep SEC 2000, 3000 and 4000, Phenomenex, Torrance, CA, USA). The columns had exclusion limits for native proteins of 300, 700 and 2000 kDa, for the SEC 2000, 3000 and 4000, respectively. A series of detectors was used to determine changes in the aggregation of the samples, with a UV detector (UV 2000, ThermoFisher) operating at 280 nm, a refractive index detector (Optilab REX, Wyatt Technology, Santa Barbara, CA, USA) and a light scattering photometer (MALLS DAWN EOS, Wyatt Technology) with a He–Ne laser (632 nm) and 18 detectors operating at various angles.

The advantage of using the SEC-HPLC with MALLS is that molecular mass and radius may be determined independently, regardless of the separation power of the columns. These measurements are not biased by sample impurities as large aggregates are easily separated from small aggregates using chromatography. The elution data were processed using ASTRA (version 5.1.9.1, Wyatt Technology). Molecular mass and mean square radius were determined directly from the absolute light scattering measurements by solving Eq. (1)

$$K^*C/R(\theta) = (16\pi^2 n^2/3M_w\lambda_0^2)\langle r_g^2\rangle \sin^2(\theta/2) + 1/M_w \quad (1)$$

where K^* is equal to $[4\pi^2 n^2 (dn/dc)^2]/(\lambda_0{}^4 N_A)$, C is the solute concentration, $R(\theta)$ is the excess intensity of scattered light at the angle θ, n is the refractive index of the solvent, λ_0 is the wavelength of incident light in a vacuum, N_A is Avogadro's number, dn/dc is the refractive index increment, M_w is the weight average molecular weight, and $\langle r_g^2\rangle$ is the mean square radius of the molecule. The average values of molecular mass and $\langle r_g^2\rangle$ were estimated from Eq. (1) using the experimental values collected during chromatographic elution for all volume slices under each peak.

Molecular mass averages are reported as $M_w = [\Sigma c_i M_i/\Sigma c_i]$, where c_i is the concentration of polymer at an elution volume i and M_i is the mass at i. From the $\langle r_g^2\rangle$ value, a z-average root mean square radius $\langle r_g^2\rangle^{0.5}{}_z$ was derived as

$$\langle r_g^2\rangle_z^{0.5}\left(= \left[\sum(c_i M_i \langle r_g^2\rangle_i^{0.5}\right)\Big/\sum(c_i M_i)\right] \quad (2)$$

This is the distribution of the volume elements of the molecule with respect to the square of the distance from the centre of gravity.

The RI detector was used as a concentration detector. A dn/dc value of 0.180 was used for the calculations of the protein peaks (Folta-Stogniew & Williams, 1999; Wyatt, 1993) whilst a dn/dc of 0.142 was used for the κ-carrageenan peak (Chambers, Langdon, Williams, & White, 1994; Singh & Jacobsson, 1994), and the aggregate peak of the mixtures. Significant differences ($p < 0.05$) were determined by analysis of variance, using the general linear model procedure, the least square means, and Duncan means using SAS (SAS Institute, Version 8).

3. Results and discussion

SEC-MALLS has been successfully employed to characterise the average molecular mass and size distribution of aggregates of β-lg or WPI formed during heating (Hoffmann et al., 1997; Kazmierski & Corredig, 2003; Schokker et al., 2000). Only a few reports are available on the use of this technique for mixed systems (Beaulieu et al., 2005; de la Fuente et al., 2004). Typical SEC-MALLS responses of the WPI solutions before heating are shown in Fig. 1. The elution signal from the UV detector clearly shows two peaks eluting at 36 and 37 min, corresponding to β-lg and α-la, respectively

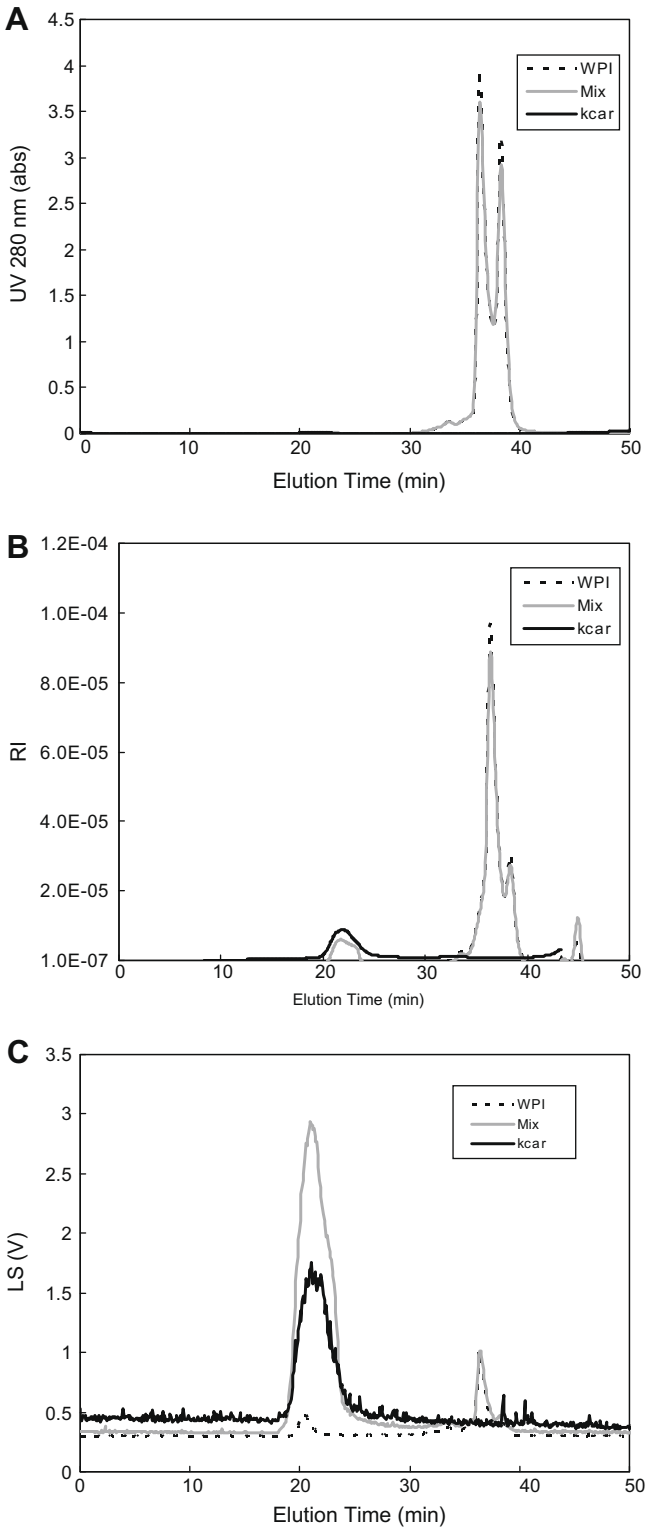

Fig. 1. Chromatography profiles for unheated solutions, WPI (0.5% w/v), κ-carrageenan (0.1% w/v) and mix (0.5% WPI and 0.1% κ-carrageenan), measured using UV (A), RI (B) and light scattering (C) detectors. Light scattering signal is shown for detector 11 (90° angle).

(Fig. 1A). No differences were noted in the UV chromatogram when unheated WPI was eluted in the presence of κ-carrageenan (Fig. 1B). The RI signal of the eluted mixture clearly showed two whey protein peaks, and an additional peak eluting at about 20 min, corresponding to κ-carrageenan. No peak was shown for unheated WPI at 20 min. The light scattering detector showed one large peak eluting at 20 min (Fig. 1C), and most of the signal in the unheated mixture could be attributed to κ-carrageenan.

The average mass (M_w, estimate of the whole peak) calculated for the β-lg peak was about 25–30 kDa, as the peak included a small shoulder containing dimeric whey protein, as already shown in earlier reports (Hoffmann et al., 1997; Schokker et al., 2000). Calculations on the peak eluting at 20 min, for unheated solutions of κ-carrageenan and κ-carrageenan mixtures with WPI, showed a M_w (weighted average for the whole peak) of $6.2 \times 2\ 10^5$ g/mol and a *z*-average root mean square radius of 87×17 nm (calculated as average of 6 separate solutions). These values are in agreement with previously reported studies (Croguennoc et al., 2001). Although a higher light scattering peak was noted in unheated mixtures of κ-carrageenan with WPI compared to the same peak for κ-carrageenan in isolation, no statistical difference was found in the average molecular mass calculated for these samples.

To obtain accurate information for the low heating temperatures, the light scattering detector was kept at the highest sensitivity setting in the DAWN EOS. However, this resulted in overload of the light scattering signal of the aggregated peak, for samples heated at 80 °C for prolonged times. Fig. 2 illustrates the UV and light scattering signals for samples heated at 75, 80 and 90 °C for 10 min, for both WPI solutions and WPI mixtures with κ-carrageenan. The UV traces clearly indicate a much higher extent of denaturation for the samples heated at 90 °C, with noticeable decreases of the unaggregated β-lg and α-la peaks eluting at 36 and 37 min for both WPI and WPI mixtures (Fig. 2A, B). A peak eluting at 20 min was also noted in the elution of heated WPI (Fig. 2A), indicating the presence of soluble protein aggregates. In WPI and κ-carrageenan mixtures, the UV peak eluting at 20 min was broader than that for WPI heated in isolation. Proteins heated at 90 °C showed no aggregate peak, indicating that the much larger aggregates formed at this temperature were eliminated during sample filtration. Light scattering (LS) traces (Fig. 2C, D) showed an increase in the LS signal for samples heated at 80 °C compared to those heated at 75 °C, and no signal for samples heated at 90 °C. A much more polydisperse signal was noticed in the case of mixed solutions compared to the WPI solutions heated in isolation.

Whilst, at short heating times, the concentration peak signal was too low to allow accurate measurements of molecular mass for the aggregates, at prolonged heating times the light scattering peak quickly reached the sensitivity limit of detection for the instrument. Therefore only samples heated at 75 °C, and some at 80 °C, could be accurately used for the calculations of molar mass and radius of the large aggregate peaks.

Fig. 3 summarises differences in the elution behaviour of samples heated at different temperatures for various times, and the effect of the presence of κ-carrageenan on the formation of intermediate aggregates. The figures focus on the shoulder at the high molecular mass end (eluting between 30 and 34 min), which corresponds to small aggregates of whey protein. This shoulder, as previously reported by Schokker et al. (1999, 2000), consists of intermediate aggregates of whey proteins and, as shown in Fig. 3, the UV signal for the aggregates eluting between 30 and 34 min increased with heating time, for both WPI solutions and mixtures with κ-carrageenan. It has been previously hypothesised that, at low concentrations of polysaccharide (below the phase separating regime), the aggregation of whey proteins proceeds unperturbed and the structure and size of the intermediate aggregates show no difference from those of whey proteins heated in isolation (Croguennoc et al., 2001). There was a clear difference in the aggregate peak eluting at 20 min, with a clear increase in the concentration of the aggregates with increasing heating time (Fig. 3). On the other hand, very similar elution patterns were shown for the intermediate aggregates eluting between 30 and 34 min. These

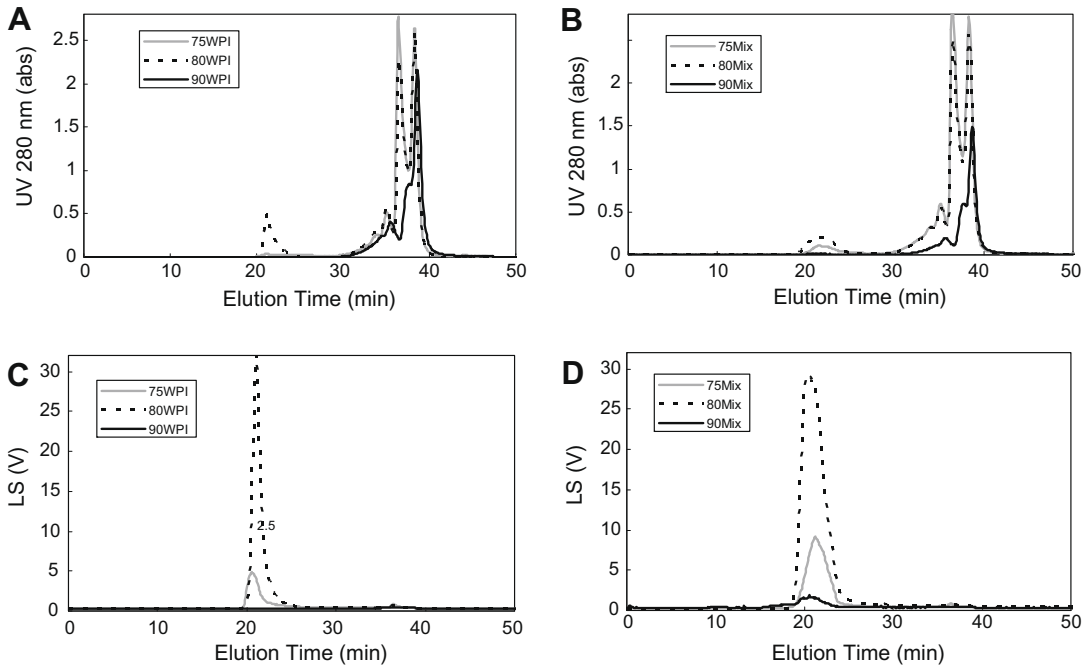

Fig. 2. Chromatography profiles for solutions of WPI heated in isolation (A, C) or in the presence of κ-carrageenan (B, D) as detected by UV and light scattering. Samples heated at 75, 80 and 90 °C for 10 min.

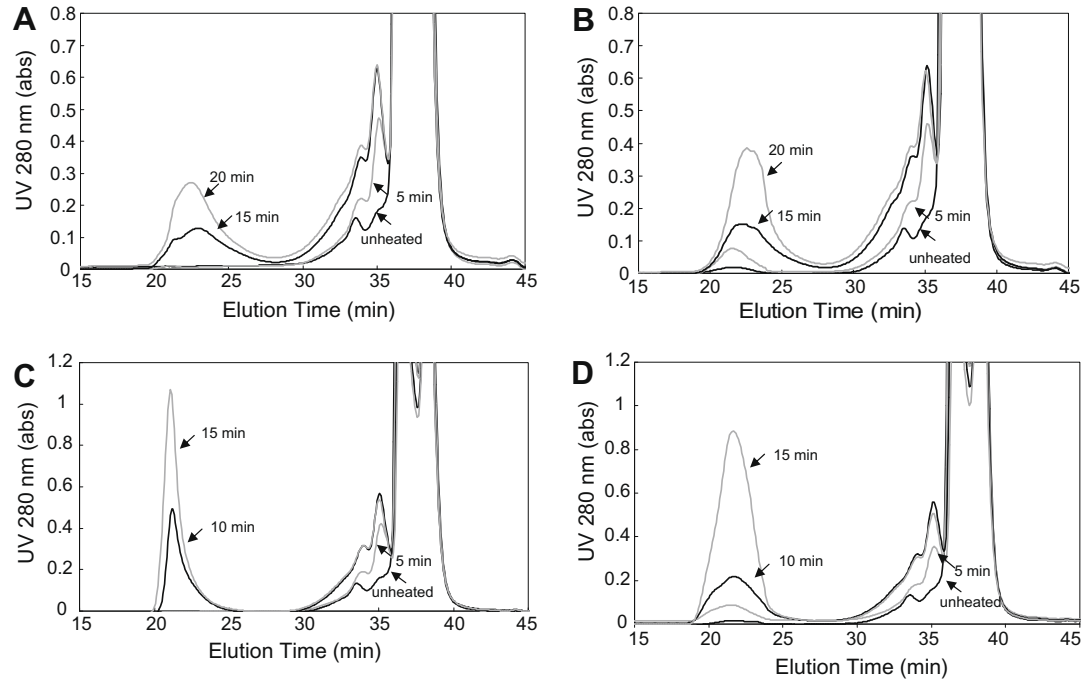

Fig. 3. UV profiles for solutions of WPI heated in isolation (A, C) or in the presence of κ-carrageenan (B, D) at 75 (A, B) or 80 °C (C, D) for various times as indicated by arrows in the graph. Note the difference in the *y*-axis between samples heated at 75 and 80 °C.

similarities existed for whey proteins heated in isolation or in the presence of κ-carrageenan (Fig. 3).

Figs. 2 and 3 clearly show that there was a decrease of residual unaggregated protein with increasing heating time and temperature. This peak includes not only native protein, but also heat-induced metastable monomers, still eluting as unaggregated protein. The amount of residual native protein was determined using SEC by measuring the area corresponding to the unaggregated protein peak (eluting at 36 and 37 min for β-lg and α-la, respectively). Fig. 4 summarises the decrease in the relative concentration (C_t/C_0) for the unaggregated β-lg and α-la. As previously reported in the literature, heating at 75 °C results in very little denaturation of whey proteins after 20 min (Schokker et al., 2000). Hoffmann et al. (1997) showed a decrease of 20% of the initial protein after 10 h of heating at 65 °C. Results shown in Fig. 3 show very little change in the unaggregated peak for samples heated at 75 and 80 °C up to

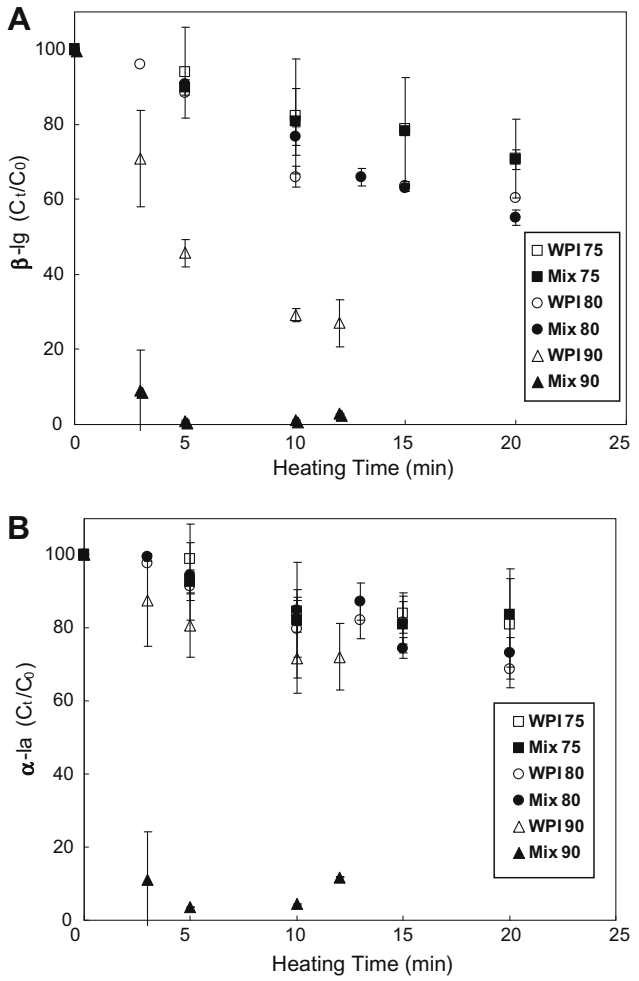

Fig. 4. Decrease (%) in the relative concentration (C_t/C_0, as calculated using RI) in the unaggregated peak for β-lactoglobulin (A) and α-lactalbumin (B) as a function of heating time. WPI solutions were heated at 75, 80 and 90 °C, either in isolation (WPI, open symbols) or in the presence of κ-carrageenan (Mix, filled symbols).

20 min. These results are in disagreement with those reported earlier (Kazmierski & Corredig, 2003), where 10 min of heating at 75 and 85 °C was sufficient to cause extensive denaturation of both β-lg and α-la. This discrepancy may be caused by the low concentration of protein employed in the present study.

Fig. 4 shows that the amounts of residual changes in the unaggregated β-lg and α-la after heating were not affected by the presence of κ-carrageenan when the mixtures were heated at 75 and 80 °C. On the other hand, heating at 90 °C showed extensive denaturation of β-lg, since a much lower amount of β-lg was recovered when WPI was heated in the presence of κ-carrageenan. In addition, for WPI heated at 90 °C in isolation, α-la does not seem to denature as fast as does β-lg, but, in the presence of κ-carrageenan, the amount of unaggregated α-la recovered was very low. This suggests an increased incorporation of α-la into the WPI aggregates when WPI is heated at 90 °C in the presence of κ-carrageenan, and a different mechanism of aggregation of WPI because of thermodynamic incompatibility between the two polymers.

A d*n*/d*c* value of 0.180 was used to determine the concentration of whey proteins in all peaks for WPI solutions, as well as all the peaks eluting >30 min for all samples, WPI solutions and mixes. On the other hand, a d*n*/d*c* value of 0.142 was used for the determination of the concentration of the peaks eluting at 20 min in the samples containing κ-carrageenan and WPI. This implies that, for the mixed solutions, the larger the amount of protein in the aggregate, the greater is the underestimation of the molecular mass. It can therefore be assumed that the differences in molecular mass between the mixes and the WPI heated in solution are underestimated. This represents a limitation in the use of light scattering to determine accurate estimates of molecular mass in mixed systems when the composition of the aggregates may change. Fig. 5 illustrates the estimated molar mass of the aggregates after heating at 75 and 80 °C for WPI heated alone or in the presence of κ-carrageenan. The presence of κ-carrageenan did not affect the molecular weight average and distribution for aggregates eluting at 20 min or the intermediate region between 30 and 34 min when heated at 75 °C. On the other hand, an obvious difference can be noted for samples heated at 80 °C in the intermediate region peak, as WPI aggregates in the presence of κ-carrageenan were larger in mass. These results are in disagreement with those reported for WPI solutions heated in the presence of polysaccharide (Beaulieu et al., 2005; de la Fuente et al., 2004). In these studies, WPI was heated in the presence of κ-carrageenan or pectin at 75 °C and at high protein concentration (compared to the present study) and a difference in the molecular mass distribution were noted between WPI heated in solution and WPI heated in the presence of polysaccharide. The molecular mass of the aggregate peak shown in Fig. 5 is in agreement with previously reported values for heated aggregates of WPI, in the range 1.6 to 4.5×10^6 Da, when heated between 65 and 85 °C (Kazmierski & Corredig, 2003).

It is important to note that, whilst at 80 °C no differences are seen in the UV elution pattern for the unaggregated peak and the intermediate peaks (Fig. 3), as well as the amount of unaggregated

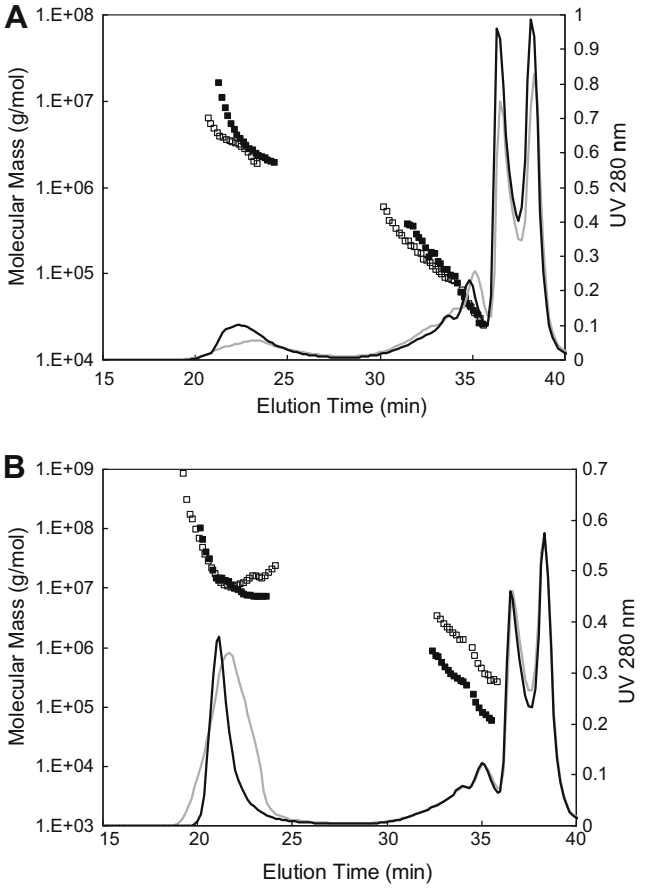

Fig. 5. Estimated molecular mass of aggregates after heat treatment for 20 min at 75 (A) and 80 °C (B) for WPI solution (empty symbols, black) and WPI-κ-carrageenan mixture (filled symbols, grey line). UV profile is also shown for solution (black line) and mix (grey line).

protein (Fig. 4), the aggregated peak was much wider in the presence of the polysaccharide. Fig. 6 summarises the values of weighted average molecular mass (M_w) for the aggregate peak (eluting at 20 min) and the two intermediate regions (elution between 32–34 and 34–36 min). Heating at 75 °C showed no differences in the molecular mass of the aggregates formed during heating, with or without κ-carrageenan. On the other hand, heating at 80 °C showed significant differences, suggesting a change in the mechanism of aggregate formation in the presence of κ-carrageenan. Although the UV signal did not suggest a significant change in the concentration of the aggregates eluting at 32–34 min, the molecular mass and *z*-average radius increased significantly (Figs. 6B,C and 7B) in the presence of the polymer. There was a clear indication of a change in the size of the aggregate peaks eluting at 32 min (Fig. 7B), as their size increased from 20 nm at the short heating time, to much larger sizes after heating for 15 min in the presence of polysaccharide. The increase in size of the aggregates, with very little change in the concentration of the unaggregated peak, may suggest a change in the shape of the intermediate aggregates in the presence of polysaccharide.

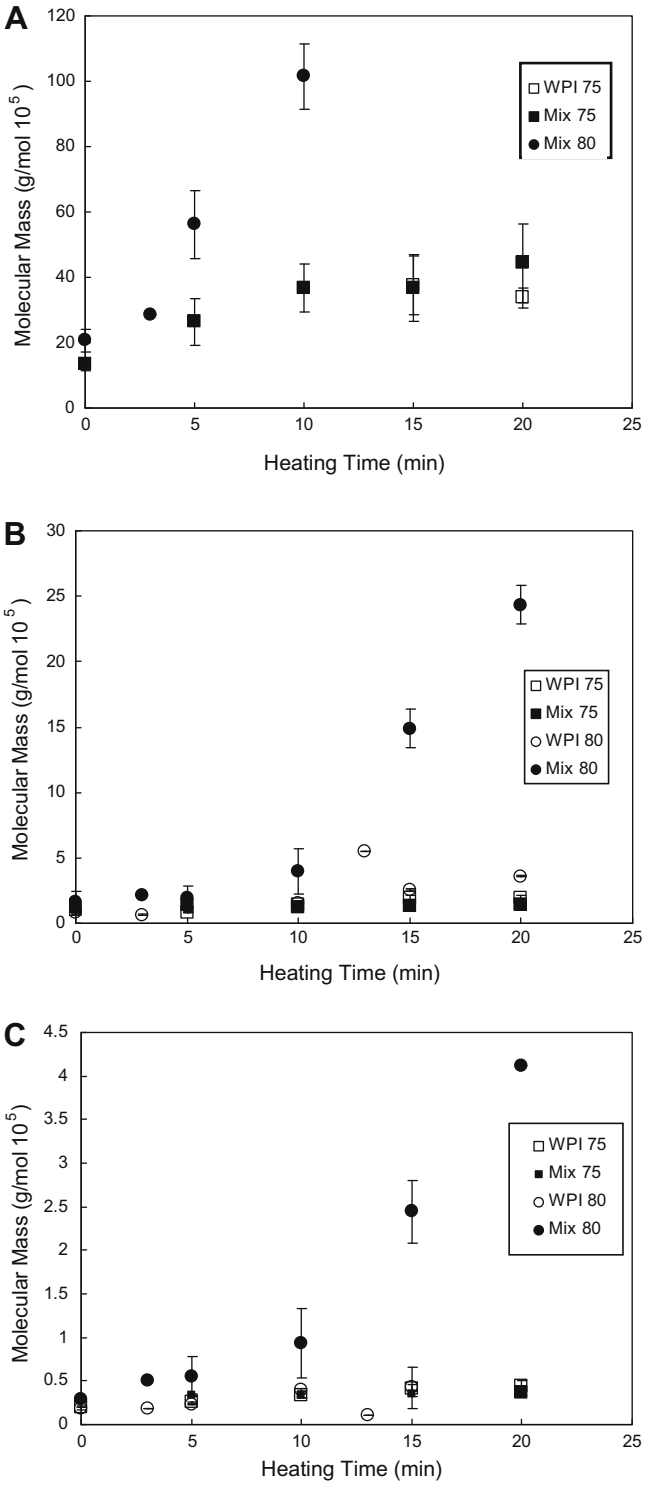

Fig. 6. Estimated average molecular mass (as M_w) for the aggregate peaks eluted at 20 min (A), 32–34 min (B) and 34–36 min (C) for WPI solution heated in isolation or in the presence of κ-carrageenan. Note that in (A) values for 80 °C WPI are not included as light scattering peak was off scale.

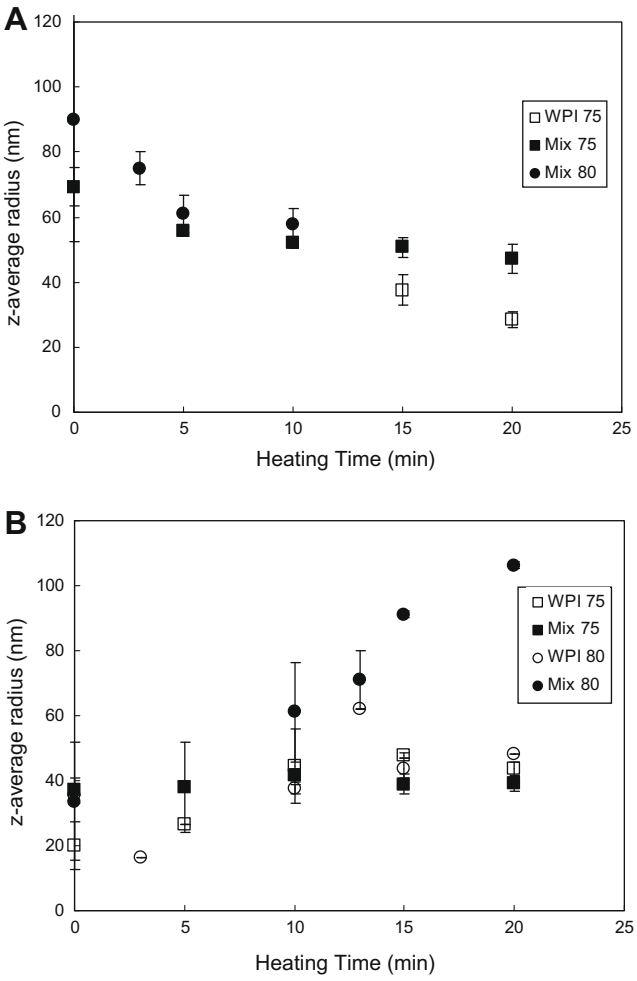

Fig. 7. *z*-Average root mean square radius for the aggregate peak eluted at 20 min (A) and at 32 min (B). Note that, in (A) values for 80 °C, WPI are not included as light scattering peak was off scale.

The size of the large aggregates (eluting at about 20 min) also changed in the mixtures compared to the WPI heated in isolation. The root mean square radius showed a decrease from about 80 to 40 nm with increasing heating time for the aggregate peak (peak eluting at about 20 min) (Fig. 7A), suggesting that more whey protein aggregates co-eluted with κ-carrageenan at longer heating times.

4. Conclusions

It has previously been hypothesised that, for a given κ-carrageenan concentration, the heat-induced aggregation of β-lg is not

affected by the polymer present in solution, at least until the aggregates of protein reach a certain size and phase separation occurs (Croguennoc et al., 2001). The present results indicate that, although this may be true for low concentration regimes and low temperatures of heating, with increasing temperature, the presence of κ-carrageenan affects the aggregation behaviour of whey proteins in solution. Although visible phase separation does not occur, the polysaccharides and whey proteins show thermodynamic incompatibility, and this affects the formation of the intermediate building blocks of the aggregation. At 90 °C, a clear effect of κ-carrageenan was noted in the amount of residual whey protein eluted in the unaggregated peak, with differences in the amount of α-la incorporated in the aggregates, clearly showing a change in the composition in the presence of polysaccharide. SEC-MALLS is a very useful technique for showing the changes in the aggregates, especially those in the 10^5–10^6 Da range. However, the technique is limited for the accurate determination of molecular mass in mixed aggregate peaks, as possible compositional changes would affect the calculation of concentration of the polymers in the peak.

Previous authors already identified an effect of κ-carrageenan during heating of WPI. Heating a 2% WPI mixture with 0.01% κ-carrageenan at pH 7.0 caused the aggregate mixture to be smaller (2.88×10^6 instead of 3.3×10^6) (de la Fuente et al., 2004). However, no consideration was given to the changes occurring in the intermediate range. A much larger effect was noted in this work when observing the changes in the molecular mass of the aggregates in the heated WPI and κ-carrageenan mixtures. We suggest that the presence of polysaccharide causes the formation of larger and more polydisperse intermediate aggregates, modifying the make-up of the final building blocks of the structure of WPI gels.

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