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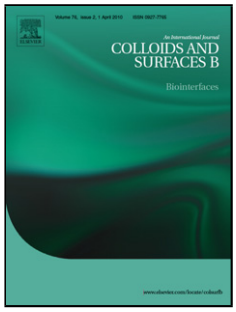

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Colloidal properties of protein complexes formed in β-casein concentrate solutions as influenced by heating and cooling in the presence of different solutes

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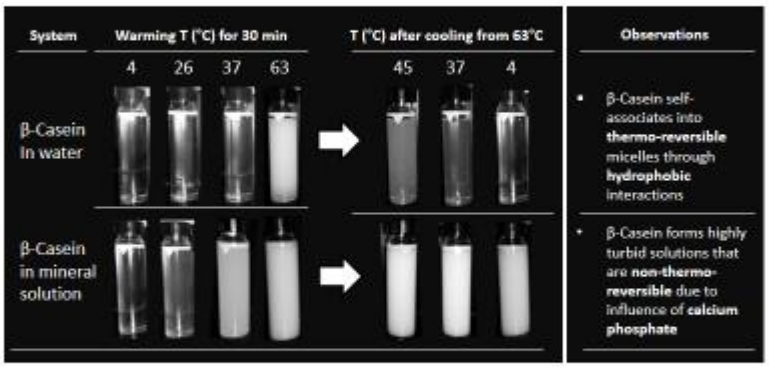

Highlights

- Self-association of a β-casein ingredient in different solutions was studied
- β-Casein formed thermo-reversible micelles in almost all systems
- Behaviour was altered in supersaturated calcium phosphate (CaP) solutions
- Formation of stable CaP phase contributed to cross-bridging of β-casein
- β-Casein-CaP systems scattered more light and was less thermo-reversible

Abstract

Monomeric bovine β-casein self-associates into micelles under appropriate conditions of protein concentration, serum composition and temperature. The present study investigated self-association characteristics of a β-casein concentrate (BCC) prepared from milk at pilot-scale using membrane filtration. The BCC had a casein:whey protein ratio of 77:23, where

~95% of casein consisted of β-casein, with the remainder being κ-CN. BCC was reconstituted to 1.2% protein (a typical level in infant formula) in various liquid media at pH 6.8 and incubated at different temperatures from 4-63°C for 30 min. Self-association of β-casein on heating was thermo-reversible in deionised water, lactose (4, 6 or 8%) or calcium (9 mM) solutions. In most serum phases, BCC became highly opaque after incubation at 63°C, but clarified rapidly during cooling to 25°C. However, in simulated milk ultrafiltrate (SMUF), which has a high ionic strength and is supersaturated in calcium phosphate (CaP), BCC remained opaque during cooling to 25°C, and retained residual turbidity after 15 h of holding at 4°C; if SMUF was prepared without phosphate then turbidity development in BCC solutions was markedly reduced. The complexes responsible for this turbidity development were successfully dissociated with 50 mM trisodium citrate. Analysis of pH during heating and holding at 60°C indicated that SMUF acidified continuously under the period of study, while acidification in BCC/SMUF mixtures terminated after a short period, indicating that the type of CaP formed on heating is altered in the presence of BCC. This study demonstrates that BCC ingredients exhibit pronounced temperature-dependant changes in colloidal properties that are strongly affected by the presence of minerals commonly found in nutritional product formulations.

1. Introduction

The caseins (CNs) of bovine milk are a heterogeneous group of four phosphoproteins (α_{s1}-, α_{s2}-, β- and κ-CN). In bovine milk, the CNs self-assemble into a polydisperse population (size range of ~ 50-500 nm, mean ≈ 150-180 nm) of colloids termed casein micelles, which are stable in the conditions of high ionic strength (80 mM) and soluble

calcium level (9 mM) which prevail in the system (O'Mahony and Fox, 2013). The CNs associate into micelles primarily through a combination of hydrophobic and electrostatic interactions, the former occurring directly between hydrophobic regions of the CNs and the latter mediated by "nanoclusters" of calcium phosphate (CaP) (Horne, 1998). The nanoclusters, often referred to as colloidal calcium phosphate (CCP), are sequestered in an amorphous form between phosphorylation sites located at hydrophilic regions of the CNs; this process prevents precipitation of CaP, which is supersaturated in milk, and facilitates delivery of significant dietary CaP without pathological precipitation in the mammary gland during milk secretion (Horne, 2006; Dalgleish, 2011; Holt and Carver, 2012; Holt, 2016).

Among the CNs, κ-CN has the lowest extent of phosphorylation (one residue), giving it a low Ca-sensitivity and reducing its potential as a nucleation site for CCP; hence, κ-CN is thought to terminate a polymerisation process that would otherwise continue indefinitely, and is generally agreed to occupy a position at the micellar surface where it stabilises micelles through electrostatic and steric repulsion (O'Mahony and Fox, 2013). The more Ca-sensitive CNs, β-CN and the α_s-CNs, contain the prerequisite number of phosphorylated residues (≥3) for binding of CCP and are mostly orientated towards the interior of the CN micelle (Dalgleish, 2011; Holt, 2016).

Removal of significant quantities of CCP through, for example, addition of calcium-binding agents, can result in non-selective dissociation of all CNs from the micellar framework (Griffin *et al*., 1988). The casein micelles of skim milk are responsible for its white colour, and removal of CCP with concomitant dissociation of the micellar structure causes a considerable decrease in the turbidity of milk, where micelles and CCP function as major light-scattering species (Smiddy *et al*., 2006). On the other hand, if milk is cooled to <5°C, the influence of hydrophobic interactions is minimised while partial dissolution of CCP occurs, resulting in 'cold-induced dissociation' of a limited quantity of monomeric β-

CN into the serum phase, while the integrity of the micellar framework is maintained (Rose, 1968; Creamer *et al*., 1977; Dalgleish, 2011).

β-CN has a high proportion of hydrophobic amino acids and this strongly affects its tendency to self-associate in solution. In the absence of native CN micelles, β-CN forms micelles with a mean diameter of 20-30 nm when above its critical micelle concentration (CMC) and at temperatures >4°C (Leclerc and Calmettes, 1997; O'Connell *et al*., 2003; Portnaya *et al*., 2006). These two phenomena, (1) cold-induced dissociation of β-CN from casein micelles and (2) self-association of β-CN in systems devoid of casein micelles, can both be exploited in the separation of β-CN from the other CNs and the separation of β-CN from whey proteins, respectively, using membrane filtration technology (Coppola *et al*., 2014; O'Mahony *et al*., 2014; Crowley, 2016).

As an ingredient, β-CN has many potential applications, including some, such as foaming and emulsification, which exploit its very high surface activity (Le Meste *et al*., 1990; Coppola *et al*., 2014). More recently, a growing body of evidence supports the application of β-CN as an encapsulating agent for hydrophobic compounds of nutritional and pharmaceutical relevance (Shapira *et al*., 2010a, 2010b, 2012; Esmaili *et al*., 2011; Bachar *et al*., 2012; Semenova *et al*., 2012; Razmi *et al*., 2013; Turovsky *et al*., 2012). Furthermore, β-CN is the dominant CN in human milk, which contains 3.87 g L^{-1} β-CN compared to only 0.77 g L^{-1} α-CN and 0.14 g L^{-1} κ-CN (Claeys et al., 2014), making β-CN-enriched protein ingredients promising candidates for humanising the CN fraction of next-generation infant formulae (McCarthy *et al*., 2013). In several respects, CN micelles in human milk are markedly different from those in bovine milk, being smaller, more hydrated and more prone to cold-induced dissociation as a few examples (Sood *et al*., 1997).

Certain factors affecting the self-association of β-CN, such as pH and temperature, or the presence of urea, sugars, denatured whey protein, or plant proteins (e.g., napin), have been studied (Yong and Foegeding, 2008; O'Connell *et al*., 2003; Schwartz *et al*., 2015; Setter and Livney, 2015). Holt *et al*. (1996, 1998) demonstrated that hydrophilic fragments of β-CN could stabilise supersaturated solutions of CaP, while Van Kemenade and De Bruyn (1989) and Thachepan *et al*. (2010) have reported similar findings for whole β-CN; however, other than these studies, little research has been carried out into the self-association of β-CN in supersaturated solutions of CaP, despite the significant role of CCP in influencing the structure and physicochemical properties of native CN micelles (Holt and Horne, 1996; Horne, 1998; Horne, 2006; Holt and Carver, 2012). The results of previous studies, which were heavily focused on the physico-chemistry of the mineral phase, have suggested that intact β-CN (Van Kemenade and De Bruyn, 1989; Thachepan *et al*., 2010) and β-CN phosphopeptides (Holt *et al*., 1996, 1998) can stabilise amorphous CaP. No self-association studies have yet included β-CN materials generated using membrane filtration, one of the most viable process technology options for the manufacture of β-CN commercially. In the present paper, such a material is studied in order to develop a better understanding of how the serum phase affects the self-association and dissociation characteristics of β-CN-based ingredients.

The purpose of this study was to explore the effects of minerals and lactose on the self-association and physical stability of β-CN concentrate (BCC), with a particular focus on the behaviour of BCC in simulated milk ultrafiltrate (SMUF), a mineral solution which is supersaturated in calcium phosphate, the precipitation of which is promoted by heating (Spanos *et al*., 2007). Although β-CN has been reported to undergo thermo-reversible micellisation, it was expected that the reversibility of this phenomenon might be markedly affected by interactions of the protein with calcium phosphate. BCC generated at pilot scale

according to a process described by Crowley (2016) was used. The process did not involve the use of precipitation, instead relying on thermo-reversible changes in β-CN's association-state, which ensures that the solubility of β-CN is maintained. Association properties of BCC were investigated in a range of serum phase compositions and temperatures (4-63°C). A protein content of 1.2% was studied, which is representative of levels found in human milk and infant formula. The results of this study could inform the use of BCCs in food and pharmaceutical applications, in which the controlled formation of micelles with specific properties (e.g., size, structure, thermal stability) may be of importance.

2. Materials and methods

2.1. Materials

β-CN concentrate (BCC) was manufactured according to the 'cold-then-warm 1 (CTW1)' process described in detail by Crowley (2016), using pressure-driven separation with spiral-wound polymeric membranes in general accordance with the process described earlier by O'Mahony *et al*. (2014). Briefly: pasteurised, skimmed milk was microfiltered and diafiltered (with milk ultrafiltration permeate) using a 0.08 µm pore-size membrane at 1.5°C to separate β-CN and whey proteins from casein micelles; the β-CN/whey protein mixture was concentrated with a 10 kDa ultrafiltration membrane at <10°C, followed by extensive demineralisation through diafiltration with reverse osmosis (RO) water at 19°C; the demineralised concentrate was warmed to 26°C to form micelles of β-CN which could then be separated from whey proteins using microfiltration (0.08 µm); the liquid BCC was further concentrated/demineralised by microfiltration/diafiltration prior to drying with a single-stage spray-drier with nozzle atomisation (PSD 55; APV, Copenhagen, Denmark). BCC powder

was stored in air-tight bags at 4°C immediately after manufacture and analysed within 6 months.

Lactose monohydrate and $Na_3C_6H_5O_7.2H_2O$ were provided by Fisher Scientific (Fair Lawn, NJ, USA). The following salts were used to prepare simulated milk ultrafiltrate (SMUF) according to the method of Jenness and Koops (1962): $CaCl_2.2H_2O$, K_2SO_4, KCl, KH_2PO_4 (Fisher Scientific, Fair Lawn, NJ, USA) and $K_3C_6H_5O_7.H_2O$, K_2CO_4 and $Mg_3(C_6H_5O_7)_2.9H_2O$ (Sigma-Aldrich, St. Louis, MO, USA). SMUF was prepared fresh for each experiment by adding salts individually to deionised water. The SMUF was then left to stir at 22°C for 60 min. After this period, drop-wise addition of >35% w/w HCl was carried out until pH ~ 3.0 was reached. The solution was stirred for a further 60 min to ensure complete solubilisation of salts, particularly CaP. The solution was then restored to pH 6.8 by drop-wise addition of 2 N (small adjustment) and 10 N (large adjustment) KOH. After bringing to volume the SMUF was inverted several times in a stoppered flask and used as a dispersant for BCC powder within 1-2 h.

2.2. Compositional analysis

BCC was analysed for total solids, protein and casein using standard procedures (AOAC, 2003). Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was performed to determine the individual CN profile (Bobe *et al*., 1998; Bonfatti *et al*., 2008). Minerals were measured using inductively-coupled plasma mass spectrometry (Herwig *et al*. 2011).

2.3. Preparation of β-casein concentrate solutions

BCC was reconstituted in a number of different liquid media, which were deionised water, lactose (4, 6 or 8%), SMUF, SMUF containing 4, 6 and 8% lactose, SMUF with a 3-

fold increase in calcium (SMUF-HC), and SMUF containing no phosphate (SMUF-NP). SMUF is a complex mineral solution which approximates the salts system of milk serum and consists of 9 mM L^{-1} Ca, 12 mM L^{-1} P, 3.2 mM L^{-1} Mg, 18 mM L^{-1} Na, 39 mM L^{-1} K, 32 mM L^{-1} Cl (Jenness and Koops, 1962). In all cases, BCC was added slowly to the dispersant over the course of 5-6 h under gentle mixing to prevent caking of the powder at the liquid surface. Once all of the powder had been added, the system was let rehydrate for a further 2 h, at which point the beaker of solution was sealed and stored at ~4°C overnight to ensure complete rehydration of the protein. The rehydration temperature used was ~22°C in most cases; however, BCC was rehydrated in SMUF at ≤15°C, unless stated otherwise, as rehydration temperature was observed to influence self-association. A completely clear BCC solution could be formed at ≤15°C; however, a temperature of 22°C resulted in irreversible turbidity development, which caused greater turbidity development during subsequent incubation experiments. The BCC solutions had a native pH of 6.7-6.9, and were adjusted to pH 6.8 if necessary with drop-wise addition of 0.1 N HCl or 0.1 N NaOH under constant magnetic stirring.

2.4. Self-association of protein in β-casein concentrate solutions

2.4.1. Effect of serum composition and incubation temperature

Refrigerated BCC solutions (30 mL at pH 6.8) were transferred to 50 mL plastic graduated centrifuge tubes and equilibrated to a solution temperature of 20°C. The centrifuge tubes were then submerged in a water bath and incubated at 26, 37 or 63°C (±0.5°C) for 30 min, unless otherwise indicated. At the specified time, samples were quickly removed from the water bath and transferred to glass, screw-capped tubes (30 mL capacity) and analysed for turbidity within 30-60 s using a nephelometer (Hach, model 2100N) with a tungsten light-

source (870 nm) and a 90° detector that measured scattered light. The nephelometer was calibrated using Formazin standards of 20, 200, 1000 and 4000 nephelometer turbidity units (NTU), with sample readings reported in NTU. No dilution was performed prior to turbidity analysis to maintain the protein content, association state and serum environment; the only exception to this was dissociation experiments, which are discussed in Section 2.4.2.

Immediately after incubation, BCC solutions were held in the glass tubes and turbidity was monitored during cooling from 63°C to 45, 37 and 26°C. BCC solutions were also analysed for turbidity at 4°C before incubation and at 4°C after 15-16 h refrigeration post-incubation; comparison of these two values gave an indication of the thermo-reversibility of the aggregation process.

2.4.2. *Dissociation of complexes in β-casein concentrate solutions*

BCC was rehydrated in SMUF at 22°C, which yielded a more turbid BCC/SMUF system. These solutions were then analysed for turbidity immediately after being incubated at 37°C for 30 min or after post-incubation holding at 4°C for 15 h. Dissociation of complexes was studied through dilution (1:1) with deionised water, SMUF or tri-sodium citrate (TSC, 100 mM) added at the same temperature as the sample to be analysed (4 or 37°C).

2.5. Size of complexes in β-casein concentrate solutions

The particle size distribution of BCC solutions prepared in deionised water or SMUF were determined using dynamic light-scattering (DLS); BCC/SMUF was prepared at 15°C to

ensure that no turbidity development occurred prior to incubation. BCC/SMUF was incubated at 4, 26, 37 or 63°C as described previously, with an additional incubation at 20°C, and samples were then diluted 1:10 in SMUF before being syringe-filtered (0.22 µm pore-size). The filtered solution was then analysed at 4, 20, 25, 35 or 60°C by DLS with a back-scattering angle of 173° using a Malvern Zetasizer nano ZSP (Malvern Instruments, Worcestershire, UK); BCC/water solutions incubated at 4 and 25°C were also analysed. Three scans were performed for each measurement. Data collection and analyses were performed using DTS (Nano) software (Version 5.02; Malvern Instruments), with the temperature-dependence of solvent viscosity being factored into size calculations. Data was transformed from intensity-weighted distributions to volume-weighted distributions using protein and solvent refractive indices of 1.45 and 1.33, respectively. It should be noted that only *apparent* hydrodynamic diameters can be measured with the Zetasizer for solvated diffusing particles with diameters larger than 60 nm that are non-spherical and polydisperse.

2.5. *Sedimentation behaviour of complexes in β-casein concentrate solutions*

An analytical centrifuge (LUMISizer®, L.U.M. GmbH, Berlin, Germany) was used to study the sedimentation of protein complexes in select samples. Two main experiments were performed; *constant incubation time*, in which the sedimentation of BCC/water and BCC/SMUF were compared with milk protein concentrate 35 (MPC35, also prepared in SMUF) after incubation for 30 min at different temperatures, and *constant incubation temperature* in which BCC/SMUF was analysed after incubation at 37°C for 90, 150 or 210 min. The principle of the instrument has been described in detail previously (Crowley *et al*., 2014). A cycle of two centrifuge speeds was applied at a controlled temperature of 37°C, the first at 36 *g* for 10 min followed by 2300 *g* for 60 min. The lower centrifugation speed

allowed the measurement of light transmission before protein sedimentation (T_{start}). Protein sedimentation occurred at the higher speed, and the clarification rate could be calculated by fitting a straight line as follows:

$$CR = \frac{T}{t+c} \quad \text{(eq. 1)}$$

where *CR* = clarification rate (%T h^{-1}), *T* = transmission (%), *t* = time (h) and *c* = intercept [T = CR(t) + c]

The final transmission value after 60 min at 2300 *g* was denoted as T_{end} and compared to T_{start} to determine the extent of sedimentation during the measurement.

2.6. *Formation of calcium phosphate during incubation*

SMUF and BCC/SMUF solutions were supersaturated with respect to CaP. At pH 6.8, an elevation of temperature should increase the degree of supersaturation and result in the formation of calcium phosphate (CaP) particles. The process can be represented as follows for the formation of tri-calcium phosphate (Lewis, 2010):

$$3Ca^{2+} + 2HPO_4^{2-} \leftrightarrow Ca_3PO_4 \downarrow + 2H^+$$

As seen by this reaction, the formation of CaP on incubation should be accompanied by a reduction in pH, meaning that acidification can be used to track the reaction, as demonstrated previously by Spanos *et al*. (2007) for SMUF at 60°C. Acidification in SMUF and BCC/SMUF solutions were monitored continuously during incubation at ~60°C. First, a probe was submerged in 10 mL of solution, with pH and temperature being measured at room temperature for 30 s; this was followed by heating to and holding at 60°C, and then cooling

back to room temperature over a period of 10 min. A holding time of 40 min was used for BCC/SMUF, but this was extended to 50 min for SMUF to investigate if steady-state pH would be reached. Straight lines were fitted to key stages to determine acidification rates. Hysteresis of pH (*ΔpH*) was also measured, to determine the reversibility of the acidification, as follows:

$$\Delta pH = pH_{start} - pH_{cooled} \text{ @ } 25°C \qquad \text{(eq. 2)}$$

with pH_{start} and pH_{cooled} indicating the pH measured before incubation and after cooling, respectively. Temperature and pH data were logged continuously during these experiments using Hach Smartlogger II v1.0.14 software (Hach, Loveland, Colorado, USA). A BX51 light microscope (Olympus, Tokyo, Japan) was used to assess if crystallisation of CaP occurred during heating OF these solutions.

2.7. *Turbidity development and protein stability on extended incubation*

Turbidity of BCC/SMUF solutions was determined as described in Section 2.4, except that 14 solutions (seven time-points, n = 2) were incubated at 37°C for up to 210 min and removed at 30 min intervals for immediate analysis of turbidity. A separate experiment was performed where BCC/SMUF incubated at 90, 150 and 210 min were incubated under the same conditions before storage at 4°C for 15 h in sealed containers; these solutions were then centrifuged at 10,000 *g* for 30 min at 10°C. The supernatant, after separation from the pellet, was diluted 1:20 and absorbance measured at 280 nm using a spectrophotometer. The absorbance values were then compared with an unincubated BCC/SMUF solution to estimate protein loss caused by incubation.

2.8. Levels of water, inorganic and organic matter in centrifugal pellets

The centrifugal pellets obtained as described in Section 2.7 were drained of supernatant upside-down for ~30 min and stored in sealed containers at 4°C for ~48 h. The pellets were kept intact as a solid mass and frozen at -20°C after removing any remaining drops of supernatant with filter paper. After ~48 h, the pellets were thawed at 4°C and weighed before being segmented into mm-sized chunks with a spatula. Individual pellet segments were then analysed using thermo-gravimetric analysis (TGA) with a TGA 500 (TA Instruments Ltd., UK). Segments of ~ 2 mg on platinum pans were transferred to an inert nitrogen-flushed atmosphere and heated (10°C min^{-1}) from ambient temperature to 550°C with weight loss from the sample being continuously monitored. Weight loss was characterised by two major stages: vaporisation of water up to 200°C and decomposition of organic matter up to 550°C. In this way, the content of water and ash could be measured for the pellets, with organic matter calculated as follows:

$$Organic\ matter\ (\%) = 100 - [water\ (\%) + ash\ (\%)] \quad \text{(eq. 3)}$$

As there was negligible organic material in the SMUF and 97% of dry-matter in the BCC powder was protein, the organic material was taken to consist primarily of protein derived from the BCC (i.e., the contribution of citrate was considered to be negligible). The yield of insoluble solids, protein and ash were calculated as follows:

$$Yield\ of\ insoluble\ material\ (\%) = \frac{Quantity\ in\ pellet\ (mg)}{Quantitiy\ in\ 30\ mL\ solution\ (mg)} \times 100 \quad \text{(eq. 4)}$$

3. Results

3.1. Composition of β-casein concentrate

The BCC powder studied consisted of 96% total solids, with 97% protein on a solids-basis, and a CN:whey protein ratio of 77:23, which approximates that of milk. RP-HPLC analysis indicated that >95% of the CN fraction was comprised of β-CN, with the remainder of the CN being composed primarily of κ-CN (4.3%). The reconstituted solution contained 1.2% protein and 0.88% β-CN. Trace levels of α_s-CN were detected (0.5% of CN), which represented <0.01% of reconstituted BCC solution on a w/v basis. In addition to protein, there was also a contribution from minerals in the composition of BCC. The Ca and P contents of the BCC powder studied were 4880 and 4342 mg kg^{-1}, respectively, giving a molar Ca:P ratio of 1.11. This would give a molar Ca concentration of 1.61 mM L^{-1} and a molar P concentration of 1.74 mM L^{-1} when the BCC was reconstituted in water.

3.2. Effect of temperature and serum-phase composition on turbidity development

At all serum compositions, turbidity of BCC increased with increasing temperature after incubation for 30 min (Fig. 1), with no visible precipitation or sedimentation observed. The contribution of whey proteins to turbidity development was considered negligible as whey protein isolate solution (0.3%, w/w, protein, i.e., total whey protein level in reconstituted BCCs) prepared in either water or milk ultrafiltrate had turbidity values of <10 NTU after incubating at temperatures between 25-63°C for 30 min (data not shown). Indeed, temperatures of >70°C are typically required to cause extensive denaturation/aggregation of whey proteins (Wijayanti *et al*., 2014). Turbidity development in the temperature range studied can therefore mainly be attributed to self-association of the CNs.

Compared to BCC/water systems (Fig. 1A), increases in turbidity were far more pronounced in BCC/SMUF (Fig. 1B), as the latter has a much higher ionic strength (~80 mM) and contributes 9 mM calcium, both of which promote β-CN self-association (Dauphas

et al., 2005). SMUF is also supersaturated in CaP, which supersaturation increases at elevated temperatures (Spanos *et al*., 2007), and can contribute to increased light-scattering when complexed with CN as nanoclusters (Smiddy *et al*., 2006). In BCC/SMUF samples, an increasing level of lactose from 0 to 4% resulted in increased turbidity at 37°C, but this effect was not observed for 6 and 8%, which seemed to slightly inhibit turbidity development. Although no clear concentration-dependant trend was observed, lactose did seem to influence development of turbidity to a minor degree. Setter and Livney (2015) reported that other sugars (glucose, galactose, mannose) altered the micellisation of β-CN, due to associated changes in water structure and hydrophobic interactions. Adjustments in the mineral profile of SMUF were made to distinguish between the influence on turbidity of ionic Ca and CaP nanocluster formation. In Figure 1C it can be seen that increasing Ca content in SMUF to ~27 mM caused turbidity development to increase strongly BCC/SMUF-HC compared to BCC/SMUF, while when BCC was prepared in SMUF with no phosphate (BCC/SMUF-NP), turbidity development was less marked. These data suggest that CaP nanocluster formation and its effect on CN self-association has a greater influence than cross-bridging of CNs by Ca ions alone.

It should be noted that heating solutions of SMUF in the absence of BCC resulted in an increase in turbidity; however, the increase was modest compared to that of BCC/SMUF solutions and extensive mineral precipitation was also observed (not evident for BCC/SMUF samples). If the turbidity of solutions of SMUF without protein are taken as a proportion of BCC/SMUF turbidity the following data are obtained: 14% at 5°C, 5% at 37°C and <8% at 63°C. Thus, the instability of SMUF is not sufficient to explain the turbidity data for BCC/SMUF samples in Figure 1; however, the form adopted by CaP complexes in SMUF compared to BCC/SMUF is likely to be different (discussed in Section 3.7), so it should be

noted that the SMUF data are not a fair representation of the contribution of CaP to turbidity in BCC/SMUF systems.

3.4. Influence of temperature on the size of protein complexes

The particle size distributions of BCC/SMUF solutions after incubation for 30 min at various temperatures are shown in Figure 2. At 4°C, BCC/SMUF samples primarily contained particles of <10 nm, with a mean value of ~6 nm, indicative of monomeric β-CN (O'Connell *et al*., 2003). BCC/water at this temperature contained particles in a similar size range, albeit slightly smaller on average. At 20°C, a shift to larger sizes of 10-60 nm (mean ≈ 20 nm) was evident for BCC/SMUF, indicative of the formation of β-CN micelles (O'Connell *et al*., 2003); a slight shoulder in the peak at ≤10 nm suggests that monomeric species remained at these temperatures. It should be noted that, although the casein micelles in bovine milk have a mean diameter of ~150 nm, the micelle size distribution is between 50 and 500 nm (O'Mahony and Fox, 2013); furthermore, the micelles formed in pure β-CN solutions are much smaller in size, at ~24 nm (O'Connell *et al*., 2003). Thus, use of the term "micelle" in the present paper refers broadly to these two types of micelle, which are both formed by the self-association of casein monomers but can result in different size distributions. Unlike BCC/water, in which a monomer/micelle mixture predominated, neither monomers nor micelles were detected in BCC/SMUF at 25°C; instead, a broad population of much larger particles (~50-400 nm) was observed. At 25, 37 or 63°C the overall shape of the particle size distribution profile in BCC/SMUF was largely unchanged, with average sizes (~140-190 nm) that are much larger than those of traditional core-shell β-CN micelles (≈ 24 nm).

3.5. Influence of temperature on the physical stability of protein complexes

Clarification profiles for BCC/water and BCC/SMUF solutions obtained from analytical centrifugation experiments are shown in Figure 3. MPC35/SMUF was included to compare the separation of β-CN micelles with native CN micelles (e.g., micelles present in milk). The results indicate negligible light-scattering in BCC/water solutions at both temperatures, with no evidence of a sedimenting population of particles for this sample. For solutions incubated at 37°C (Fig. 3A), BCC/SMUF had a reduced transmission compared to BCC/water at the early stages of centrifugation; this effect was especially pronounced after incubation at 63°C, and was in agreement with turbidity data (Fig. 1). The change in transmission with centrifugation time for the BCC/SMUF sample incubated at 37°C was relatively constant and slightly uneven (Fig. 3A); this unusual profile was due to a combination of sedimentation phenomenon (top half of cell) and continued self-association of β-CN (bottom half of cell), as can be seen more clearly in the space- and time-resolved profile in Figure S1 (supplementary files). This effect was not observed for BCC/SMUF incubated at 63°C, for which a very low T_{start} (~10%) was detected, and which displayed almost complete sedimentation of particles within 2000 s of centrifugation (Fig. 3B). In contrast, the sedimentation behaviour of MPC35 was unaffected by incubation temperature, as native CN micelles are not subject to such dynamic and dramatic shifts in association-state under these conditions.

3.6. Thermo-reversibility of turbidity development

Samples which had been incubated at 63°C were subjected to cooling to 22°C on the bench to investigate the degree to which turbidity development during incubation was

thermo-reversible (Fig. 4). For samples incubated at 63°C, it took 5, 15 and 40 min to reach 45, 37, and 26°C, respectively. During this time, all BCC/SMUF systems remained visually opaque (milk-like) and turbidity was greater than the measurement limit of the nephelometer (Fig. 4). Conversely, the turbidity of BCC/water systems began to decrease instantaneously during cooling and was completely reversed.

Also shown in Figure 4 is the turbidity of the systems after 15 h of cooling at 4°C; notably, all BCC/SMUF systems had retained a measurable residual turbidity level (~400-1100 NTU), while all BCC/water systems had NTU values <10. Similar, if less pronounced and consistent, trends were observed for samples incubated at 37°C (data not shown). Unlike previous reports (O'Mahony *et al*., 2014; Schwartz *et al*., 2015) these β-CN complexes were not fully thermo-reversible, or at least not within the 15 h cooling period that was investigated. The authors consider this incomplete/delayed thermo-reversion to be due to interactions between β-CN and CaP, which are highlighted in the dissociation studies reported in Section 3.7.

3.7. Dilution-induced dissociation of complexes in β-casein concentrate solutions

The finding that the thermo-reversal of turbidity development in BCC/SMUF systems is remarkably slow and ultimately incomplete (Section 3.6) indicates that the integrity of the complexes formed cannot be attributed to hydrophobic interactions alone. To investigate this further, a BCC/SMUF system was prepared at 22°C and incubated at 37°C. Samples were then analysed for turbidity at 37°C undiluted, or after 1:1 dilution in water, SMUF or tri-sodium citrate (TSC). The same experiment was performed after first cooling the incubated sample at 4°C for 15 h, with samples being analysed either undiluted or in the aforementioned diluents.

The results of warm and cold dissociation experiments are shown in Figure 5. In BCC/SMUF systems at 37°C, dilution with water resulted in an 82% decrease in turbidity. Dilution in SMUF resulted in an increase in turbidity, which placed the sample outside the measurement limit for the instrument; the solution was already very close to this limit when undiluted, and the increase in turbidity after dilution suggests that continued protein aggregation occurred after extended incubation in the presence of SMUF, despite a dilution of protein content. Dilution in TSC resulted in a change in turbidity to values close to those observed for unincubated BCC/SMUF systems at 4°C (~13 NTU). TSC has a limited impact on the refractive index of the solvent (Smiddy *et al*., 2006), and additional decreases in turbidity caused by TSC can therefore be attributed to its dissociating action (i.e., sequestering of Ca). After incubation, cooling of undiluted BCC/SMUF to 4°C resulted in a 93% reduction in turbidity, although turbidity was still quite high (252 NTU). At this temperature, dilution with water or SMUF achieved a decrease in turbidity of ~50%. The presence of TSC at 4°C caused more extensive dissociation than at 37°C, resulting in a low turbidity of 2.6 NTU. The slow thermo-reversal of this micellisation process may be attributable to the slow rate of dissolution of CCP cross-links, compared to a more rapid weakening of hydrophobic interactions. A decrease in the Ca-binding ability of caseins at low temperatures probably also contributed to observed dissociation phenomena (Horne and Lucey, 2014). The nature of the attractive and repulsive forces involved in these systems is discussed in more detail below (Section 3.10).

3.8. Formation of calcium phosphate phases during incubation

It was observed that SMUF incubated at 37 or 63°C for 30 min showed precipitation (not shown) but no precipitation occurred under these conditions in BCC/SMUF solutions.

These data suggested that the crystallisation of CaP was being prevented by proteins in the BCC. CaP formation was tracked dynamically by analysing acidification during heating to, and holding at, 60°C (Fig. 6).

Data for SMUF are shown in Figure 6A. An initial decrease from pH 6.8 to 6.5 occurred during the first 500 s of heating from 25°C, followed by a lag phase lasting ~1000 s and a second acidification stage which continued until the temperature was reduced. When subjected to cooling to the original temperature pH was partially restored. For BCC/SMUF (Fig. 6B), the initial stage of acidification was similar to that observed for SMUF. After this point, however, the two samples diverged considerably. No second acidification stage was detected for BCC/water; instead, an extended lag phase at pH 6.4 was measured. A partial restoration of pH was, again, measured after cooling for this sample.

The two-stage acidification that was a feature of SMUF was divided into stage A, which was rapid, and stage B, which was slow. Only stage A was detected in BCC/SMUF. The rate of acidification (pH unit s^{-1}, $\times 10^{-4}$) in stage A was -4 ± 0 and -8 ± 1 for SMUF and BCC/SMUF, respectively. Stage B, detected only for SMUF, had an acidification rate of -1 ± 0, indicating it was a comparatively slow phenomenon. After cooling for 10 min, a ΔpH of 0.33 ± 0.01 was measured for SMUF, while the value for BCC/SMUF was only 0.1 ± 0.01. Light microscopy images of SMUF during heating and holding indicated the presence of crystals of 20-33 μm, while no crystals were observed in BCC/SMUF (data not shown). Holt and Carver (2012) described how CaP precipitation in supersaturated solutions commences with the formation of an amorphous state that serves as a precursor phase to lower energy crystalline states (i.e., octocalcium phosphate, hydroxyapatite); in the presence of phosphoproteins, CaP formation is arrested at the amorphous stage. Thachepan *et al*. (2010) found that β-CN could stabilise CaP in the amorphous state during incubation >35°C. Results for the heat-induced acidification of SMUF and BCC/SMUF are consistent with the reports

of these previous authors and demonstrate that protein in BCC can act as a nucleation site for amorphous CaP, which prevents crystallisation and thereby limits acidification.

3.9. Turbidity development and colloidal destabilisation of protein during incubation

When BCC/SMUF was incubated at 37°C, turbidity increased over time (Fig. 7A). Irreversible destabilisation occurred for incubation periods of ≥60 min, with precipitates observed after subsequent cooling (~15 h at 4°C). When the destabilised BCC/SMUF systems were centrifuged, the supernatants were shown to have an almost 10% decrease in absorbance of light at 280 nm, indicating the presence of more insoluble protein compared to the unincubated control (Fig. 7A).

The results in Figure 7A indicate that, when BCC was incubated in SMUF for >30 min, an aggregation process was initiated which ultimately led to the irreversible destabilisation of a limited quantity of protein. This phenomenon was very likely to be heat-induced rather than cooling-induced, as demonstrated by data from analytical centrifugation experiments performed immediately after incubation at 37°C for 30, 90, 150 or 210 min (data not shown). Data from the analytical centrifuge showed a strong, positive linear relationship between incubation time and clarification rate, *CR* (R^2 = 0.99). Evaluation of light transmission through the sample at the start (T_{start}) and end (T_{end}) of centrifugation indicated that longer incubation times resulted in an initial increase in light scattering due to protein complexes (low T_{start}) and a greater change in turbidity due to sedimentation of these complexes (high T_{end}). For example, after 30 min incubation the T_{start} and T_{end} were 77.2 ± 0.0 and 77.2 ± 0.2 %, respectively, with a CR of 0.31 ± 0.1 % h^{-1}, suggesting a low initial turbidity and a negligible degree of sedimentation; on the other hand, after 210 min incubation the T_{start} and T_{end} were 61.5 ± 0.7 and 82.2 ± 0.5, respectively, with a CR of 26.6 ±

1.0 % h^{-1}, demonstrating that the longer incubation time promoted the formation of more sedimentable complexes.

Pellets obtained from centrifugation of BCC/SMUF incubated for 90, 150 and 210 min were analysed by TGA to determine levels of water, organic and inorganic constituents. Representative TGA profiles are shown in Figure 7B. Organic and inorganic material was considered to be composed of protein and ash, respectively. Increasing incubation time resulted in increases in the content of both the protein and ash in the pellets, with a notable decrease in water content between the 150 and 210 min incubation times compared to pellet from 90 min incubation (Fig. 7B). Trends in the yield of insoluble protein were generally consistent with UV absorbance at 280 nm data, with both sets of data suggesting only a limited degree of protein destabilisation (7-13%), reaching a maximum after ~150 min incubation (data not shown).

3.10. Mechanism of self-association/dissociation of β-CN in presence of CaP

Overall, the results of this study demonstrate that the self-association and dissociation behaviour of β-CN is likely influenced by multiple attractive and repulsive forces. Self-association of β-CN is promoted by heating (Fig. 1, 2, 3). Cooling-induced dissociation of β-CN micelles (Fig. 4) is due to hydrophobic interactions decreasing at lower temperatures but also due to a reduction in the calcium binding capacity of the β-CN (Horne and Lucey, 2014). This latter effect would, in turn, increase negative charge on the β-CN, which, in combination with decreased hydrophobic interactions, could be expected to cause micelle dissociation. In a system supersaturated with calcium and phosphate (i.e., SMUF, in this case), an additional consideration is the formation of a CaP phase during heating, which participates in the formation of highly turbid solutions (Fig. 1) of large micelles (Fig. 2, 3) that are less

susceptible to dissociation (Fig. 4, 5). In the absence of β-CN, CaP precipitates and ultimately forms hydroxyapatite (Holt and Carver, 2012), with the consequent release of all hydrogens resulting in considerable acidification (Fig. 6A). In the presence of β-CN, the type of CaP can be predicted to be in a different form, such as dicalcium phosphate (Holt *et al*., 1996), which would cause less H^+ to be released and much more limited acidification (Fig. 6B). In such a system, hydrophobic association between two or more β-CN molecules may leave exposed hydrophilic (charged) segments that can bind CaP, forming CaP "nanoclusters" similar to what is found in the casein micelles of milk (Horne, 2006; Dalgleish, 2011; Holt and Carver, 2012; Holt, 2016). The resultant heat-induced complexes, held together by a combination of hydrophobic interactions, ionic calcium binding and CaP cross-bridging, are more difficult and slow to dissociate by cooling (Fig. 4, 5), and a limited proportion may undergo irreversible destabilisation on extended incubation (Fig. 7). These findings are potentially relevant to future work focused on exploiting the functional properties of β-CN (e.g., encapsulation, surface activity). For example, knowledge of the influence of CaP on the association/dissociation behaviour of β-CN can contribute to strategies aimed at designing complexes that are more stable to temperature changes (with CaP) or less stable to temperature changes (just Ca).

4. Conclusion

In this study, a membrane process-derived BCC was assessed for its association and dissociation behaviour. Both the extent and reversibility of self-association were influenced by reconstitution temperature, serum-phase composition, incubation temperature and time. A

key finding was that β-CN complexes could partially stabilise a supersaturated solution of CaP; in turn, CaP promoted the formation of β-CN complexes which were larger than typical β-CN micelles and more resistant to dissociation. Irreversible, albeit limited, precipitation occurred on extended incubation of BCC in SMUF, likely due to a progression of the polymerisation process mediated by hydrophobic and ionic interactions. The nature of the association and dissociation characteristics of protein-mineral complexes in BCCs could have important implications for their use in certain applications, such as the humanisation of infant formulae, fortifying foods with bioavailable forms of minerals, encapsulation of drugs or bioactives, and the stabilisation of emulsions and foams.

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7. References

Association of Official Analytical Chemists (2003). *Official Methods of Analysis of AOAC International*, 17th edn. Gaithersburg, MD: AOAC International Publisher.

Bachar, M., Mandelbaum, A., Portnaya, I., Perlstein, H., Even-Chen, S., Barenholz, Y., and Danino, D. (2012). Development and characterization of a novel drug nanocarrier for oral delivery, based on self-assembled β-casein micelles. *Journal of Controlled Release*, 160, 164-171.

Bobe, G., Beitz, D. C., Freeman, A. E., and Lindberg, G. L. (1998). Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, 46, 458-463.

Bonfatti, V., Grigoletto, L., Cecchinato, A., Gallo, L., and Carnier, P. (2008). Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. *Journal of Chromatography A*, 1195, 101-106.

Claeys, W. L., Verraes, C., Cardoen, S., De Block, J., Huyghebaert, A., Raes, K., Dewettinck, K., and Herman, L. (2014). Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. *Food Control*, 42, 188-201.

Coppola, L. E., Molitor, M. S., Rankin, S. A., and Lucey, J. A. (2014). Comparison of milk-derived whey protein concentrates containing various levels of casein. *International Journal of Dairy Technology*, 67, 467-473.

Creamer, L. K., Berry, G. P. and Mills, O. E. (1977). A study of the dissociation of β-casein from the bovine casein micelle at low temperature. *New Zealand Journal of Dairy Science and Technology*, 12, 58-66.

Crowley, S. V. (2016). Physicochemical characterisation of protein ingredients prepared from milk by ultrafiltration or microfiltration for application in formulated mutritional products. *PhD thesis*, University College Cork, Cork, Ireland. Available online: https://cora.ucc.ie/handle/10468/3243?show=full.

Crowley, S. V., Kelly, A. L., and O'Mahony, J. A. (2014). Fortification of reconstituted skim milk powder with different calcium salts: Impact of physicochemical changes on heat- and physical-stability. *International Journal of Dairy Technology*, 67, 474-482.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. *Soft Matter*, 7, 2265-2272.

Dauphas, S., Mohous-Rious, N., Metro, B., Mackie, A. R., Wilde, P. J., Anton, M., and Riaublanc, A. (2005). The supramolecular organisation of β-casein: effect on interfacial properties. *Food Hydrocolloids*, 19, 387-293.

Esmaili, M., Ghaffari, S. M., Moosavi-Movahedi, Z., Atri, M., S., Sharifizadeh, A., Farhadi, M., Yousefi, R., Chobert, J.-M., Haertlé, T., and Moosavi-Movahedi, A. A. (2015). Beta casein-micelle as a nano vehicle for solubility enhancement of curcumin; food industry application. *LWT – Food Science and Technology*, 44, 2166-2172.

Griffin,M. C., Lyster, R. L., and Price, J. C. (1988). The disaggregation of calcium-depleted casein micelles. *European Journal of Biochemistry*, 174, 339-343.

Herwig, N., Stephan, K., Panne, U., Pritzkow, W., & Vogl, J. (2011). Multi-element screening in milk and feed by SF-ICP-MS. *Food Chemistry*, 124, 1233-1230.

Holt, C. (2016). Casein and casein micelle structures, functions and diversity in 20 species. *International Dairy Journal*, 60, 2-13.

Holt, C., and Carver, J. A. (2012). Darwinian transformation of a 'scarcely nutritious fluid' into milk. *Journal of Evolutionary Biology*, 7, 1253-63.

Holt, C., and Horne, D. S. (1996). The hairy casein micelle: Evolution of the concept and its implications for dairy technology. *Netherlands Milk and Dairy Journal*, 50, 85-111.

Holt, C., Timmins, P. A., Errington, N., and Leaver, J. (1998). A core-shell model of calcium phosphate nanoclusters stabilized by β-casein phosphopeptides, derived from sedimentation equilibrium and small-angle X-ray and neutron-scattering measurements. *European Journal of Biochemistry*, 252, 73-78.

Holt, C., Wahlgren, M., and Drakenberg, T. (1996). Ability of a β-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters. *Biochemical Journal*, 314, 1035-1039.

Horne, D. S. (1998). Casein interactions: Casting light on the black boxes, the structure in dairy products. *International Dairy Journal*, 3, 171-177.

Horne, D. S. (2006). Casein micelle structure: models and muddles. *Current Opinion in Colloid and Interface Science*, 11, 148-153.

Horne, D. S., and Lucey, J. A. (2014). Revisiting the temperature dependence of the coagulation of renneted bovine casein micelles. *Food Hydrocolloids*, 42, 75-80.

Jenness, R., and Koops, J. (1962). Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Netherlands Milk and Dairy Journal*, 16, 153-164.

Leclerc, E., and Calmettes, P. (1997). Interactions in micellar solution of β-casein. *Physical Review Letters*, 78, 150-153.

Le Meste, M., Colas, B., Simatos, D., Closs, B., Courthoudon, J. L., and Lorient, D. (1990). Contribution of protein flexibility to the foaming properties of casein. *Journal of Food Science*, 55, 1445–1447.

Lewis M.J. 2010. The measurement and significance of ionic calcium in milk – A Review. *International Journal of Dairy Technology*, 1-13.

McCarthy, N. A., Kelly, A. L., O'Mahony, J. A., and Fenelon, M. A. (2013). The physical characteristics and emulsification properties of partially dephosphorylated bovine β-casein. *Food Chemistry*, 138, 1304-1311.

O'Connell, J. E., Grinberg, V. Ya, and de Kruif, C. G. (2003). Association behavior of β-casein. *Journal of Colloid and Interface Science*, 258, 33-39.

O'Mahony, J.A., and Fox, P.F. (2013). Milk proteins: introduction and historical aspects, in, *Advanced Dairy Chemistry, Volume 1A: Proteins: Basic Aspects*, 4th edn. (P.L.H. McSweeney and P.F. Fox, (eds.), Springer, New York. pp. 43-85.

O'Mahony, J. A., Smith, K. E., and Lucey, J. A. (2014). Purification of beta casein from milk. Patent: US20070104847A1.

Razmi, M., Divsalar, A., Saboury, A. A., Izadi, Z., Haertlé, T., and Mansuri-Torshizi, H. (2013). Beta-casein and its complexes with chitosan as nanovehicles for delivery of a platinum anticancer drug. *Colloids and Surfaces B: Biointerfaces*, 112, 362-367.

Rose, D. (1968). Relation between micellar and serum casein in bovine milk. *Journal of Dairy Science*, 51, 1897-1902.

Portnaya, I., Cogan, U., Livney, Y. D., Ramon, O., Shimoni, K., Rosenberg, M., and Danino, D. (2006). Micellization of bovine β-casein studied by isothermal titration microcalorimetry and cryogenic transmission electron microscopy. *Journal of Agricultural and Food Chemistry*, 5555-5561.

Schwartz, J.-M., Solé, V., Guéguen, J., Ropers, M.-H., Riaublanc, A., and Anton, M. (2015). Partial replacement of β-casein by napin, a rapeseed protein, as ingredient for processed foods: Thermoreversible aggregation. *LWT – Food Science and Technology*, 63, 562-568.

Semenova, M. G., Antipova, A. S., Anokhina, M. S., Belyakova, L. E., Polikarpov, Yu., N., Grigorovich, N. V., and Tsapkina, E. N. (2012). Thermodynamic and structural insight into the underlying mechanisms of the phosphatidylcholine liposomes – casein associates co-assembly and functionality. *Food and Function*, 3, 271.

Setter, O., and Livney, Y., D. (2015). The effect of sugar stereochemistry on protein self-assembly: the case of β-casein micellization in different aldohexose solutions. *Physical Chemistry Chemical Physics*, 17, 3599-3606.

Shapira, A., Assaraf, Y. G., and Livney, Y. D. (2010a). Beta-casein nano-vehicles for oral delivery of chemotherapeutic drugs. *Nanomedicine: Nanotechnology, Biology and Medicine*, 6, 119-126.

Shapira, A., Davidson, I., Avni, N., Assaraf, Y. G., and Livney, Y. D. (2012). β-Casein nanoparticle-based oral drug delivery system for potential treatment of gastric carcinoma: Stability, target-activated release and cytotoxicity. *European Journal of Pharmaceutics and Biopharmaceutics*, 6, 547-555.

Shapira, A., Markman, G., Assaraf, Y. G., and Livney, Y. D. (2010b). β-Casein nano-vehicles for oral delivery of chemotherapeutic drugs: drug-protein interactions and mitoxanthrone loading capacity. *Nanomedicine: Nanotechnology, Biology and Medicine*, 6, 547-555.

Smiddy, M. A., Martin, J. –E. G. H., Kelly, A. L., de Kruif, C. G., and Huppertz, T. (2006). Stability of Casein Micelles Cross-linked by Transglutaminase. *Journal of Dairy Science*, 89, 1906-1914.

Sood, S. M., Herbert, P. J., and Slattery, C. W. (1997). Structural studies on casein micelles of human milk: Dissociation of β-casein of different phosphorylation levels induced by cooling and ethylenediaminetetraacetate. *Journal of Dairy Science*, 80, 628-633.

Spanos, N., Patis, A., Kanellopoulou, D., Andritsos, N., and Koutsoukos, P. G. (2007). Precipitation of calcium phosphate from simulated milk ultrafiltrate solutions. *Crystal Growth and Design*, 7, 25-29.

Thachepan, S., Li, M., and Mann, S. (2010). Mesoscale crystallization of calcium phosphate nanostructures in protein (casein) micelles. *Nanoscale*, 2, 2400-2405.

Turovsky, T., Khalfin, R., Kabaya, S., Schmidt, A., Barenholz, Y., and Danino, D. (2015). Celecoxib encapsulation in β-casein micelles: Structure, interactions, and conformation. *Langmuir*, 31, 7183-7192.

Van Kemenade, M. J. J. M., and De Bruyn, P. L. (1989). The influence of casein on the precipitation of brushite and octacalcium phosphate. *Colloids and Surfaces*, 36, 359-368.

Wijayanti, H. B., Bansal, N., and Deeth, H. C. (2014). Stability of whey proteins during thermal processing: a review. *Comprehensive Reviews in Food Science and Food Safety*, 13, 1235-1251.

Yong, Y. H., and Foegeding, E. A. (2008). Effects of caseins on thermal stability of bovine beta-lactoglobulin. *Journal of Agricultural and Food Chemistry*, 56, 10352-10358.

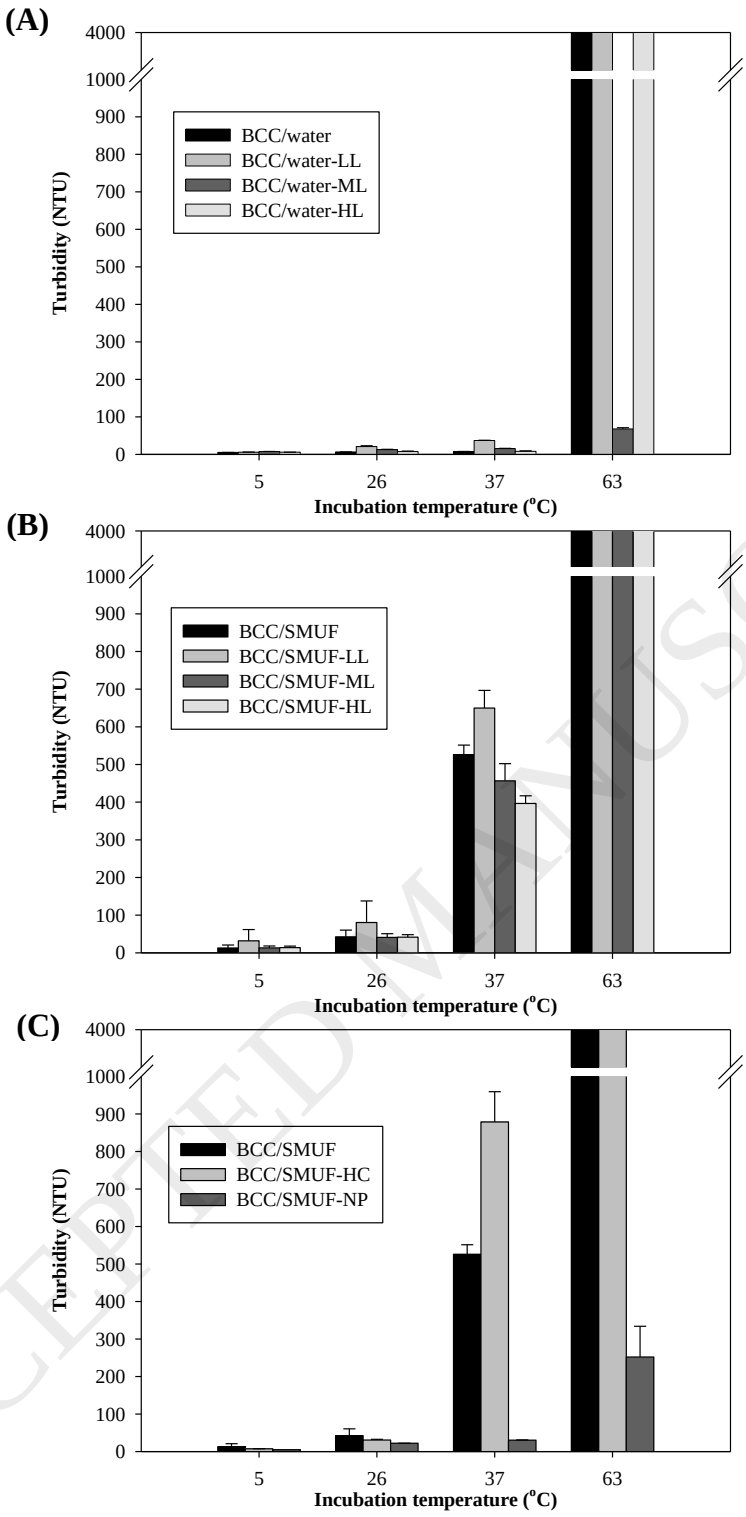

Fig. 1. Turbidity of β-casein concentrate (BCC) solutions prepared in (A) water or (B) simulated milk ultrafiltrate (SMUF) with 4% (LL), 6% (ML) or 8% (HL) lactose, and incubated at specific temperatures for 30 min. Profiles are also shown for (C) high Ca SMUF prepared with 27 mM Ca (SMUF-HC) and SMUF containing no phosphate (SMUF-NP) compared to standard SMUF. Data are the means (± standard deviations) of experiments on solutions prepared, incubated and analysed on at least two separate occasions. Detection limit = 4000 NTU.

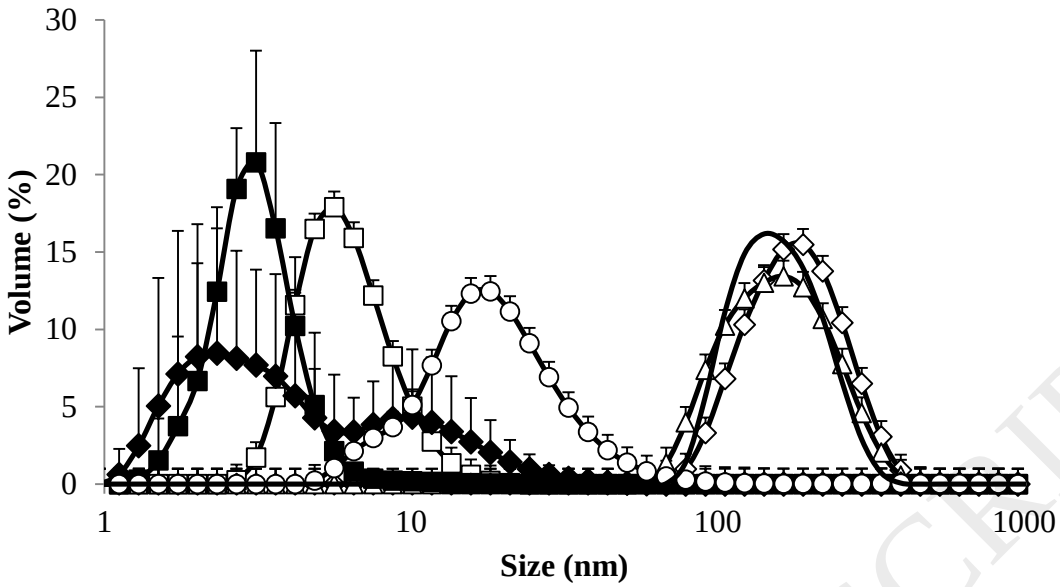

Fig. 2. Particle size distributions of β-casein concentrate (BCC) solutions prepared in simulated milk ultrafiltrate (SMUF), and incubated at 4, 20, 26, 37 or 63°C for 30 min prior to analysis; profiles generated for each at 4°C (□), 20°C (○), 25°C (◊), 35°C (Δ) or 63°C (—) are shown. Solutions were diluted (1:10) and filtered (0.22 µm) prior to analysis. Results for BCC/water solutions at 4°C (■) and 25°C (♦) are included for comparison. Data are means (± standard deviations) for solutions prepared for two separate incubation experiments, prepared and analysed for size in duplicate. The data at 63°C was the result of a single analysis on pooled filtrate due to low sample volume.

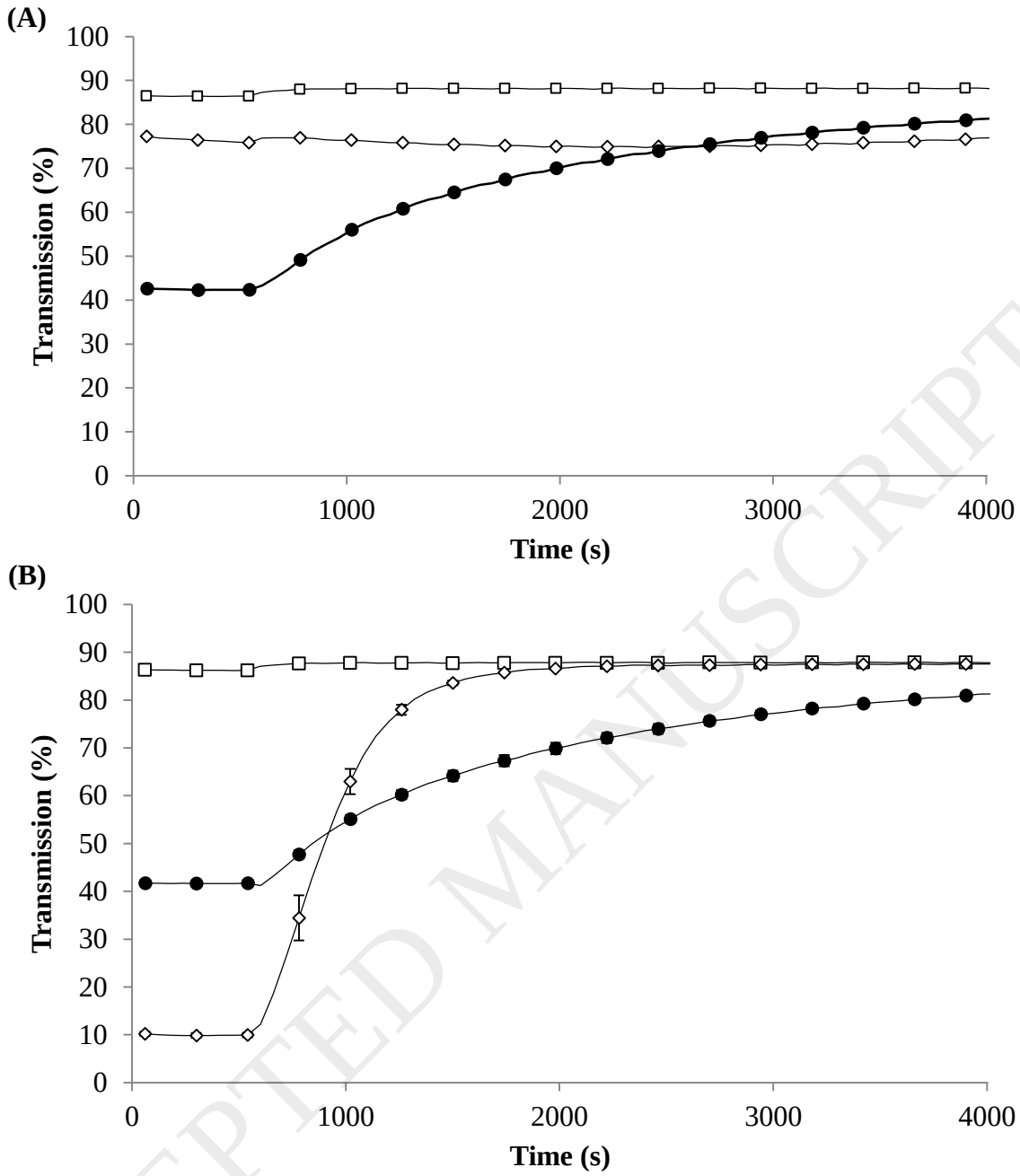

Fig. 3. Transmission of near-infrared light during centrifugation of solutions of β-casein concentrate (BCC) prepared in water (□) or simulated milk ultrafiltrate (SMUF, ◇) as a function of centrifugation time. Milk protein concentrate 35 (MPC35) prepared in SMUF (●) is shown for comparison. Solutions were incubated at (A) 37°C or (B) 60°C for 30 min before centrifugation. A cycle of two centrifuge speeds was applied at a controlled temperature of 37°C, the first at 36 *g* for 10 min followed by 2300 *g* for 60 min. Data are the means (± standard deviations) of duplicate experiments.

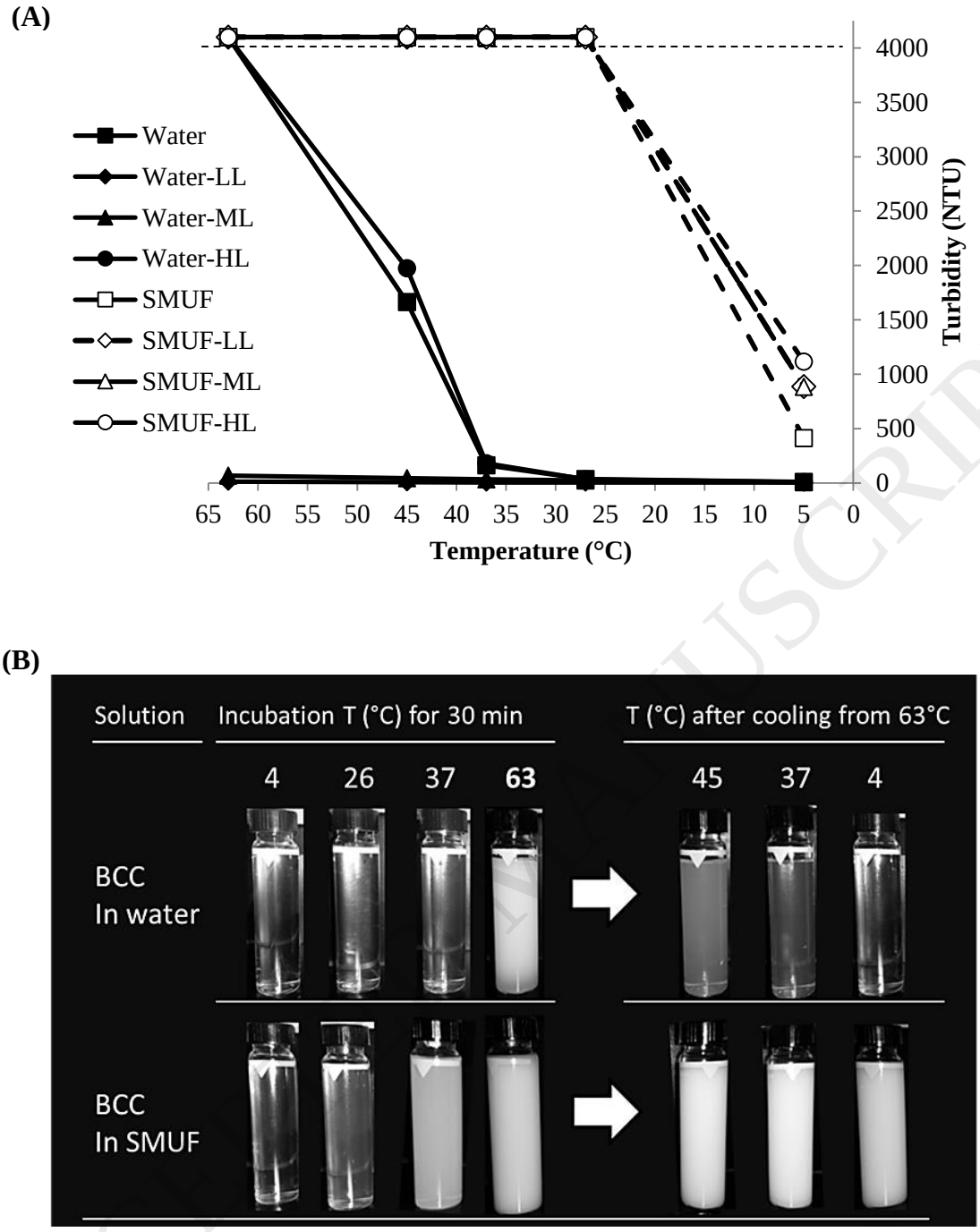

Fig. 4. (A) Turbidity of β-casein concentrate (BCC) solutions prepared in water or simulated milk ultrafiltrate (SMUF) during cooling from 63°C and (B) photographs of representative samples. Cooling to 45, 37 and 27 °C took 5, 15 and 40 min, respectively; the 5 °C samples were analysed after overnight refrigerated storage. LL – 4% lactose, ML = 6% lactose, HL = 8% lactose. Data are the means of experiments on solutions prepared, incubated and analysed on at least two separate occasions. Note: data points above the broken line indicate that the detection limit of the instrument was being exceeded.

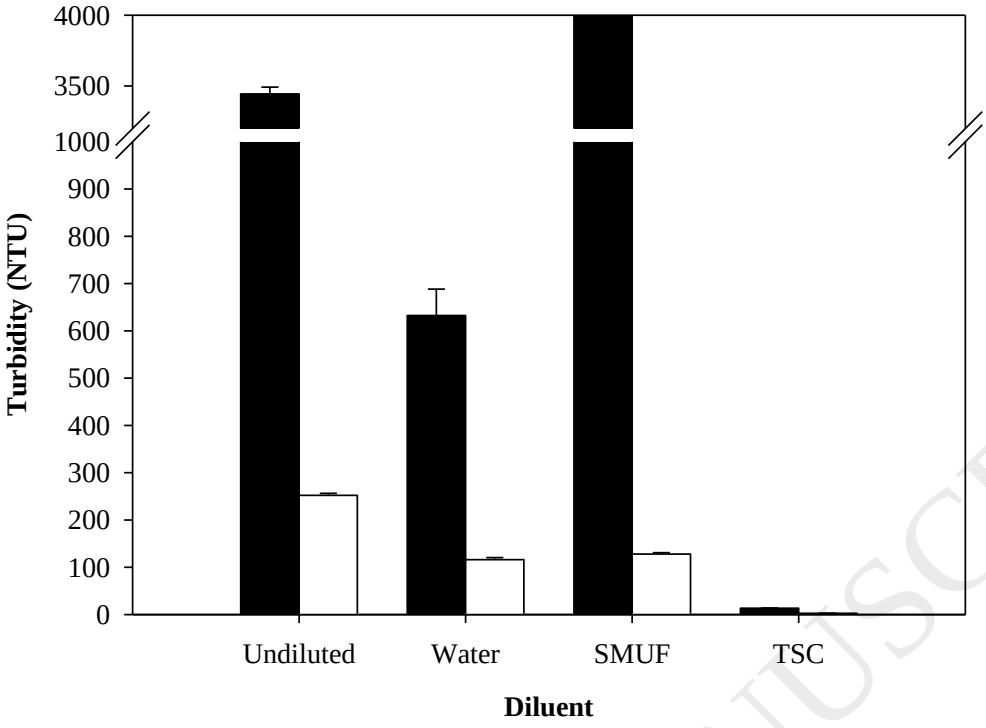

Fig. 5. Turbidity of β-casein concentrate (BCC) solutions prepared in simulated milk ultrafiltrate (SMUF) at 22°C and incubated at 37°C for 30 min followed by immediate dilution in different solvents (black bars) or dilution after holding at 4°C for 15 h (white bar). Dilutions were 1:1 in all cases. Data are the means (± standard deviations) of solutions subjected to two separate incubation/dilution/cooling experiments. Upper measurement range = 4000 NTU.

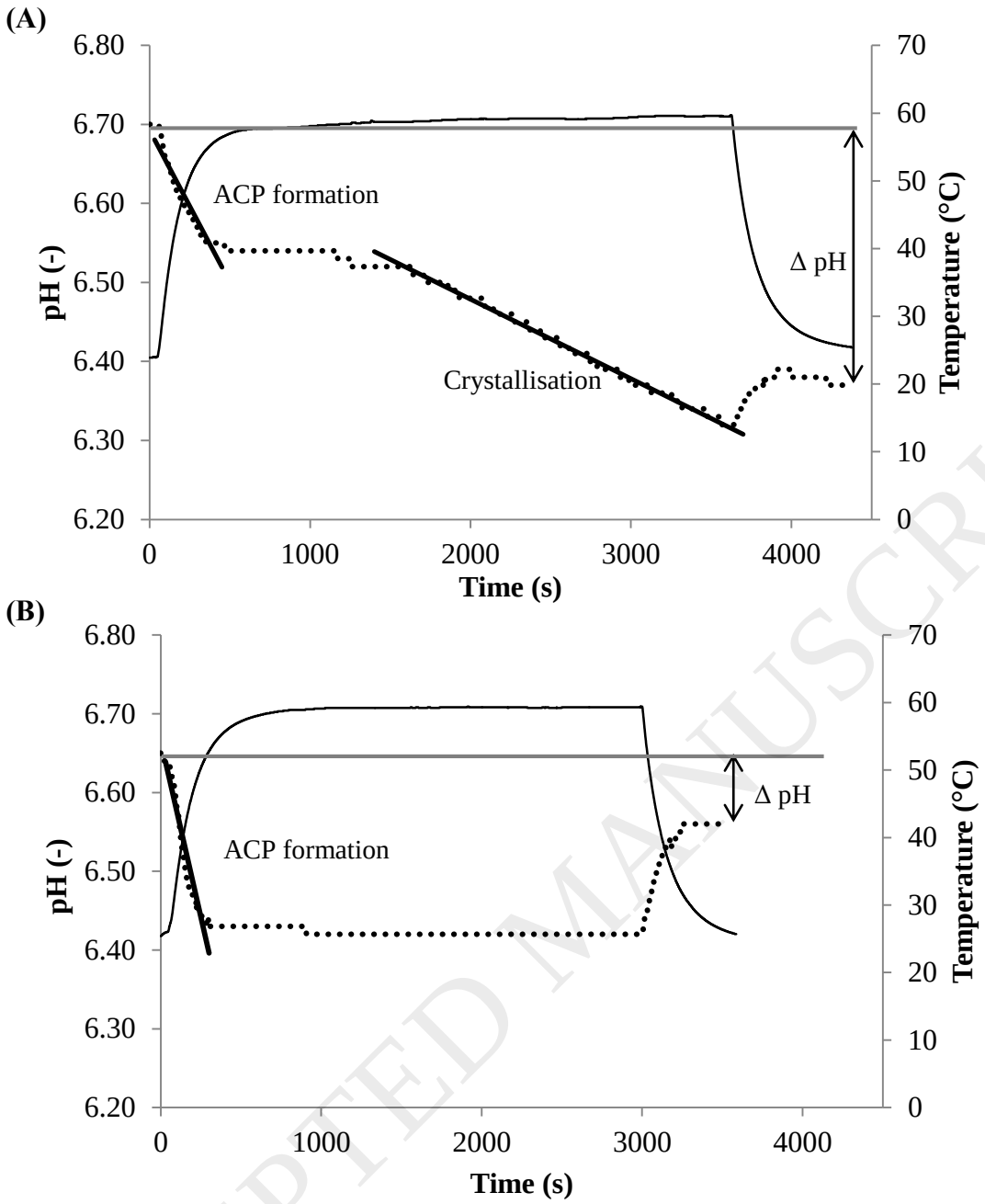

Fig. 6. Changes in pH during incubation of (A) simulated milk ultrafiltrate (SMUF) and (B) β-casein concentrate (BCC) in SMUF at ~60°C. Temperature is shown as a solid curve and pH as a broken curve. A grey horizontal line is shown extending from starting pH to guide the eye for pH hysteresis (*ΔpH*) calculation. Linear fits are fitted to pH curves to estimate the rate of amorphous calcium phosphate (ACP) formation and crystallisation. Data are from a single analysis but are representative of replicate profiles.

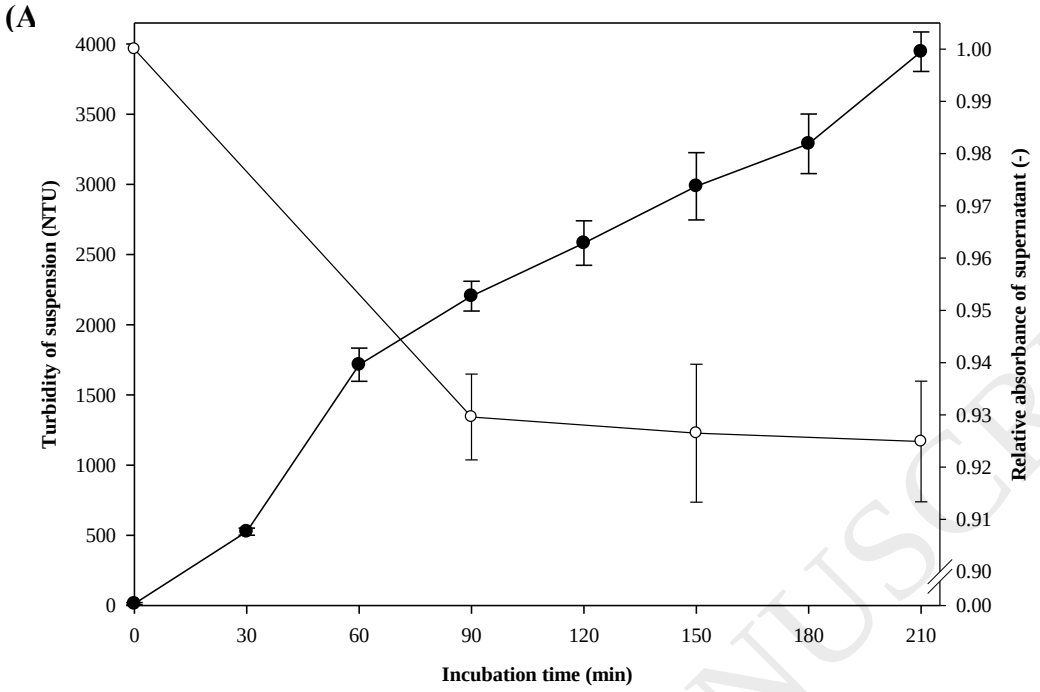

Fig. 7. (A) Turbidity of β-casein concentrate (BCC)/simulated milk ultrafiltrate (SMUF) solutions during incubation at 37°C at different times (closed symbols) and the absorbance at 280 nm of soluble protein in the supernatant after refrigeration (~15 h) and centrifugal removal of precipitated material. (B) Results of thermo-gravimetric analysis on 2.0-2.5 mg sub-samples of pellets obtained from BCC/SMUF solutions after incubation at 37°C for 90, 150 or 210 min, refrigeration for 15 h, centrifugation and removal of supernatant. Compositional data was derived from measurement of evaporative loss of water (25 to 200°C) and decomposition of organic material (200 to 550°C). Data are the means (± standard deviations) of solutions subjected to two separate incubation experiments, with supernatants/pellets generated in duplicate and prepared for analysis separately.