



Influence of casein on the formation of whey protein microparticles obtained by dry heating at an alkaline pH

Elise Schong, Marie-Hélène Famelart

► To cite this version:

Elise Schong, Marie-Hélène Famelart. Influence of casein on the formation of whey protein microparticles obtained by dry heating at an alkaline pH. Food Research International, 2019, 122, pp.96-104. <10.1016/j.foodres.2019.04.001>. <hal-02098680>

HAL Id: hal-02098680

<https://hal.science/hal-02098680v1>

Submitted on 22 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC-ND 4.0 - Attribution - Non-commercial use - No Derivative Works - International License

1 Influence of casein on the formation of whey protein microparticles obtained
2 by dry heating at an alkaline pH

3 Elise Schong, Marie-Hélène Famelart*

4 STLO, UMR 1253, INRA, Agrocampus Ouest, 35000 Rennes cedex, France

5 *Corresponding author; Tel: +33.223.48.53.43; Fax: +33.223.48.53.50; email address: marie-
6 helene.famelart@inra.fr

7 **ABSTRACT**

8 Dry heating (DH) at 100 °C for 36 h of a whey protein isolate powder conditioned at pH 9.5
9 leads to the formation of stable, large and porous whey protein microparticles (PMs),
10 resulting from the crosslinking of proteins inside the powder. These PMs could be used as
11 high-viscosity food ingredients. Casein, present as a contaminant in whey protein powders,
12 has been shown to become incorporated into the PMs. In this study, we investigated the
13 effect of adding increasing amounts of sodium caseinate to whey protein powders on the
14 formation of PMs during DH at 100 °C for 36 h. In addition, we studied PM formation during
15 DH of a micellar casein-enriched milk protein powder (Casmic). The browning index of the
16 dry-heated powders, and the size and water content of the microparticles were also
17 characterized. We confirmed that sodium caseinate was incorporated into the PMs. The
18 highest PM D[4,3] values (270 µm) were observed for powders with around 40% caseinate.
19 Powders without added caseinate displayed D[4,3] values of 150 µm. The yield of conversion
20 of proteins into PMs increased from 0.6 to 0.8 g/g with caseinate addition, whereas the
21 amount of water entrapped in the PMs decreased from around 30 to 20 g/g. PMs were also
22 formed by DH of the Casmic powder, but these particles were smaller, with sizes of around
23 80 µm. In conclusion, our study shows that the process of DH at pH 9.5 could be applied to
24 all milk proteins to obtain PMs with functional properties that could be used in the food
25 industry.

26 **KEYWORDS:** whey protein; microparticle; dry heating; aggregation; casein

1. Introduction

Whey proteins (WPs) display a range of useful functional characteristics, such as rheological, foaming, emulsifying and gelation properties (Dickinson, 2015; Kharlamova, Chassenieux, & Nicolai, 2018; T. Nicolai & Durand, 2013). A lot of work has been dedicated to the study of WP aggregation, with the aim of increasing the functional properties of these proteins. Most of these studies involved heating a solution of WPs under a range of physicochemical conditions and studying the size, structure and properties of the resulting aggregates (Taco Nicolai, Britten, & Schmitt, 2011). During the last two decades, an increasing number of studies have investigated the effect of directly heating protein powders in the presence of sugar to induce the Maillard reaction (O'Mahony, Drapala, Mulcahy, & Mulvihill, 2017; Schong & Famelart, 2017). Whereas very few changes in protein structure are observed during the glycation of proteins under gentle dry heating (DH) conditions (Fenaille, Morgan, Parisod, Tabet, & Guy, 2003), DH treatments using high temperatures and/or over long time periods can induce significant WP aggregation (Gulzar, Bouhallab, Jeantet, Schuck, & Croguennec, 2011; Hiller & Lorenzen, 2010; Schmitt, Bovay, & Frossard, 2005).

Our previous studies have shown that DH for over 10 h at 100 °C of a freeze-dried or a spray-dried WP powder conditioned at pH 9.5 with a water activity (a_w) of around 0.2 leads to the formation of WP microparticles (Famelart, Schong, & Croguennec, 2018; Schong & Famelart, 2018). By dissolving the dry-heated powder in water and observing the protein microparticles (PMs), we have shown that these PMs are very large and that their size and shape are influenced by the size and shape of the powder particles. The DH treatment led to crosslinking of proteins inside the powder particles so that the powder was no longer soluble when dispersed in water. Due to the porous structure of the powder, the PMs formed after 36 h of DH were able to entrap a huge amount of water (20-40 g water/g of dry PM), with a yield of formation of over 0.5 g of PM/g of powder. The PMs produced by DH of the spray-dried

powder were spherical with a hollow structure, and were smaller and more homogeneous in size than the those produced from DH of the freeze-dried powder (Famelart et al., 2018; Schong & Famelart, 2018). The very high viscosity values obtained in our previous study indicates that these PMs could be used as high-viscosity or food thickening agents (Schong & Famelart, 2018). The suspension of particles formed by DH of the spray-dried WP powder at 32 g/kg gave a viscosity of around 17 Pa.s at a shear rate of 1 s^{-1} and a temperature of 20 °C. This viscosity value is much higher than that obtained for other WP particles in previous studies (Dissanayake et al., 2012; Inthavong, Kharlamova, Chassenieux, & Nicolai, 2016; Loveday, Su, Rao, Anema, & Singh, 2012; Zhang, Arrighi, Campbell, Lonchamp, & Euston, 2016).

More recently, we studied the effect of lactose content on PM formation from freeze-dried powders (Schong & Famelart, 2019). Although freeze-dried flakes are much larger than spray-dried powder grains, freeze-dried powders were used as a model for spray-dried powders in this study because it was more convenient to produce powders with different lactose compositions by freeze-drying than by spray drying. Using this model, we showed that increasing the lactose content led to increases in the rate of protein crosslinking and PM formation, and to higher yields of conversion of WP into PMs to more than 0.9 g/g of powder. In contrast, increasing the lactose content led to a decrease in the amount of water entrapped in these PMs. It has been shown that dry-heated powder particles are able to swell when placed in water, leading to particle sizes in suspension being slightly larger than those in the powder. However, powder particles exposed to longer DH times and those heated in the presence of higher lactose contents swelled less when placed in water, due to the higher degree of crosslinking induced by DH. DH at 100 °C for 36 h without added lactose, but in the presence of the trace amounts of lactose present in the WP powder (around 6 g per kg powder), gave the same yield of conversion of WP into PM and led to the same amount of water entrapped in the PMs as DH for only 3 h with addition of lactose at a concentration of 0.42 g/10 g protein. Nevertheless, evaluation of the browning index of the

dry-heated powder showed that the formation of the PMs did not correlate with the progress of the Maillard reaction. Indeed, the mechanism of formation and the nature of the protein crosslinks are still speculative. We have found that the PMs are resistant to reduction (unpublished results). Similar findings were reported by Gulzar et al. (2011), who found that WP aggregates produced by DH at pH 4.5 or 6.5 were resistant to reduction, whereas aggregates produced at pH 2.5 were not. Isopeptide and lysinoalanine bonds have been implicated in the formation of the crosslinks as their formation is favored by high pH values and severe heating (Gerrard, 2002). Furthermore, large protein aggregates have been reported in the presence of lactose as a result of the Maillard reaction: these aggregates are made of covalent bonds resistant to reduction and have been reported to involve the degradation products of Maillard intermediates, such as dicarbonyl compounds (Friedman, 1996; Hiller & Lorenzen, 2010; Lan et al., 2010; Miller, Meade, & Gerrard, 2003; Zamora & Hidalgo, 2005). These dicarbonyl compounds are highly reactive crosslinkers of lysine and arginine residues and give rise to advanced glycation end products (Gerrard et al., 2005; Miller et al., 2003). Their formation is enhanced at an alkaline pH (Martins & Van Boekel, 2005; Wei, Liu, & Sun, 2018). However, the large number of reactants and the complexity of the pathways involved in the Maillard reaction, together with an inability to dissociate the PMs into smaller protein chains for analysis, mean that it appears impossible to determine the exact nature of the PM bonds.

The WP powder used in these previous studies was 90% protein. About 18% of these proteins were caseins, present as contaminants of the WP powder. It has been shown that these caseins were transferred into the PMs together with the WPs (Schong & Famelart, 2019). The aim of the current study was to further our understanding of the mechanism of PM formation by investigating the effect of replacing WPs with casein on the formation of PMs. As in our previous study investigating the effect of lactose content on PM formation, freeze-dried powders were used in the current study as a convenient model for predicting the behavior of caseins on spray-dried powders. Herein, we report on the changes in the

properties of the powder particles and PMs induced by adding sodium caseinate to WP before freeze-drying and DH at 100 °C for 36 h. To gain an insight into the effect of casein structure on its transfer into the PMs, a sample of micellar casein-enriched milk protein was also studied after undergoing the same heating process.

2. Materials and Methods

Most of the materials and methods used in this study have been described in more detail previously (see Schong & Famelart (2019)).

2.1. Materials

The commercial WP powder was obtained from a confidential source and was the same powder as that used in our previous studies (Famelart et al., 2018; Schong & Famelart, 2018, 2019). The solid matter and protein contents of the WP powder were 965.6 ± 0.3 and 911 ± 7 g per kg powder, as determined respectively by the desiccation method (International Dairy Federation, 2010), and by the Kjeldahl method (International Dairy Federation, 2004, 2014, 2016) using 6.38 as the conversion factor. Approximately 82% of this protein was WP and around 18% was casein, present as a mix of α s1, α s2, β and κ caseins, as determined by polyacrylamide gel electrophoresis (Schong & Famelart, 2019). The free-lactose content of the powder was 5.92 ± 0.16 g per kg powder as determined by ion chromatography (Gaucheron, 2003). Thus, the lactose to protein ratio was around $6.5 \cdot 10^{-3}$ g/g.

The commercial sodium caseinate (NaCas) powder was obtained from Lactalis (Bourgbarré, France) and contained 936.9 ± 3.4 g of solid matter, $878.06 \text{ g} \pm 2.1$ of protein and $2.99 \text{ g} \pm 0.03$ g of free-lactose per kg powder. Thus, the lactose to protein ratio for the NaCas powder was around $3.4 \cdot 10^{-3}$ g/g.

The commercial micellar casein-enriched milk (Casmic) protein powder (Promilk 852B) was obtained from International dairy ingredients (Arras, France) and contained 936.7 ± 0.5 g of solid matter, 779.8 ± 0.2 g of protein (among which 93% was micellar casein) and 29.42 ± 1.16 g of lactose per kg powder. The proportion of casein and WP in this powder was determined by Kjeldahl analysis of non-protein and non-casein nitrogen. The lactose to protein ratio was around 38.10^{-3} g/g, and was thus 6–11 fold higher than that for the two other powders.

2.2. Preparation of the dry-heated powders

The NaCas powder was mixed with the WP powder at levels of 0, 20, 25, 33, 50 and 100% by weight. The casein content as a percentage of the weight of total protein was calculated for each mixture based on the composition of the powders as 18, 34, 38, 44, 58 and 99%, respectively. These samples were then referred to as “Cas18” to “Cas99”. The lactose to protein ratio decreased progressively by a factor of two from the Cas18 powder to the Cas99 powder. Similarly, the dry matter content in the solution showed a 3% decrease from 965.6 to 936.9 g/kg. A sample of 100% of Casmic, containing 93% casein, was also prepared.

Solutions containing 150 g powder/kg were prepared by stirring overnight at 4 °C. The pH of these solutions was then adjusted to pH 9.5 by addition of 5 mol.L^{-1} and then 1 mol.L^{-1} NaOH. The solutions were then rapidly frozen and freeze-dried. The powders were finely crushed, adjusted to an a_w of 0.25 ± 0.01 for two weeks and dry-heated at 100 °C for 36 h. Only one solution and powder were prepared for each mixture of casein and WP, but two samples of each powder solution were dry-heated and further analyzed.

151 2.3. Analysis

152 2.3.1. Powder

153 The granular size distribution of the unheated freeze-dried powder after crushing was
154 analyzed as described previously in Schong & Famelart (2019) using the QICPIC analyzer
155 and the dry disperser RODOS/L module (Sympatec GmbH, Clausthal-Zellerfeld, Germany).
156 Two measurements of the distribution of the “diameter of a circle of equal projection area” of
157 the particles were taken for each sample and the mean volume diameter (D[4,3]) was
158 deduced. Two measurements were performed for each unheated sample.

159 Samples of powder particles were transferred to a glass lamella and observed on a light
160 microscope (Olympus BX51, Rungis, France) with a x10 objective. Photographs (10 per
161 sample) were taken using a Qiclick CCD camera (Q Imaging, Microvision Instruments, Evry,
162 France) and the Archimed software program (Microvision Instruments).

163 The intensity of the Maillard reaction was estimated by monitoring changes in the
164 browning index (BI) of the powders. Briefly, the BI was deduced from L* (whiteness), a*
165 (redness/greenness) and b* (yellowness/blueness) measurements made using a CR-300
166 Chromameter (Minolta, Roissy, France). The BI was obtained according to Maskan (2001) as
167 follows:

168
$$BI = \frac{[100 (x - 0.31)]}{0.17}$$
 equation 1

169
$$x = \frac{(a^* + 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)}$$
 equation 2

170 Three measurements were performed for each dry-heated powder.

2.3.2. Proteins and protein microparticles

Powders were reconstituted to a final concentration of 10 g of powder.kg⁻¹ in 0.2 g.kg⁻¹ sodium azide in deionized water and adjusted to pH 6.5 (suspension S0). One S0 suspension was prepared for each dry-heated powder.

Samples (1 mL) of the S0 suspension were acidified by addition of 300 µL of 0.5 M acetate-acetic acid buffer (pH 4.6), and then centrifuged for 15 min at 10000 g. The resulting supernatants, containing residual native whey proteins (NWP), were then frozen until analysis. Casein is not soluble at pH 4.6 and was partitioned into the pellet during this fractionation process. The levels of native β-Lactoglobulin (β-Lg) and α-Lactalbumin (α-lac), present in the supernatants were quantified by reversed phase high performance liquid chromatography (RP-HPLC) as described in Schong & Famelart (2019). NWPs were quantified as the sum: β-Lg + α-lac. One sample was prepared for each S0 suspension and three RP-HPLC injections were done for each preparation.

The size distribution of PMs with sizes of 0.02–2000 µm was measured in S0 suspensions at 20 °C using a Mastersizer 2000 equipped with the HydroSM module (Malvern Instruments, Worcestershire, UK), as described by Famelart et al. (2018). The D[4,3] was then deduced. Two samples were taken from each S0 suspension and measurements on each sample were carried out in triplicate. Changes in the D[4,3] of the powder particles in suspension were evaluated by determining the size ratio (SR) from the D[4,3] of the PMs/D[4,3] of the powder particles.

PMs were isolated by centrifuging around 2 g of each S0 suspension at 10 000 g for 15 min at 20 °C. Pellets containing sedimented PMs were then weighed in the wet state to obtain the wet weight (WW) and then after drying using a Savant Speed Vac Concentrator SVC-100H (Hicksville, USA) to obtain the dry weight (DW). The dry yield of conversion of the powder dry matter into PMs (DY) was then determined as the dry weight of the pellet obtained from 1 g of dry-heated powder. The wet yield of conversion (WY) was determined

as the wet weight of the pellet obtained from 1 g of dry-heated powder. As the dry matters of the WP and NaCas mixtures and of the Casmic powder were made up of about 94% and 83% protein, respectively, the DY and WY provided good estimation of the yield of conversion of proteins into particles. The water content (WC) of the PMs, determined as the weight of water per 1 g of dry pellet, was calculated as follows:

$$WC = \frac{(WW-DW)}{DW} \quad \text{equation 3}$$

Measurements were performed four times for each suspension.

S0 suspensions were diluted 10-fold in distilled water and observed by confocal laser scanning microscopy (CLSM) using an inverted TE2000-E microscope (Nikon, Champigny-sur-Marne, France) with a x20 oil-immersion objective. Proteins were probed with Nile Blue. Ten images per sample (512 x 512 pixels², representing 636 x 636 μm²) were acquired at a distance of 15 μm away from the bottom lamella.

2.4. Statistical analysis

The effects of powder composition on the D[4,3] and on the residual NWP content were analyzed by analysis of variance (ANOVA) and, in case of significance, by the Tukey's multiple comparison test for paired comparisons of means. A P value of less than 0.05 was used as the level of significance. All statistical analyses were performed using the R software package.

3. Results and discussion

3.1. Powder characterization

The BI of the powders before (0 h) and after DH for 36 h is shown in Fig. 1. For the WP powder without added casein (Cas18 sample), the BI was 12 before DH and 45 after 36 h of

DH. These values are in accordance with those described previously: BI scores of 7-16 before DH and 27-54 after 36 h of DH were obtained for the same powder after same treatments by Famelart et al. (2018) and Schong & Famelart (2018, 2019). The BI of all powders containing a mixture of WP and NaCas was around 10 before DH and increased by a factor of four after DH. The highest BI after DH was obtained for the powders with around 40% casein; the BI then decreased as the NaCas content of the powders increased. This decrease in BI may be explained by a reduction in the lactose to protein ratio when WPs were replaced by NaCas; however, the reason for the increase in BI between powders with 18% casein and those with 44% casein is unknown. One hypothesis is that the alkaline pH applied before DH induced WP denaturation and unfolding, leading to an increased exposure of the lysine residues and their glycation via the Maillard reaction. Glycation levels in caseins would be expected to be higher than those in WPs as caseins are unstructured proteins and do not need to be unfolded to be able to react with lactose. This could explain the initial increase in BI with increased casein content. The BI of the Casmic powder increased by a factor of 12 after DH, probably because this powder had a higher lactose to protein ratio promoting a more intensive Maillard reaction.

The granular size distribution of the unheated powder is shown in Fig. 2A. The size distribution of the powder particles made of WP and NaCas showed a slight shift towards larger sizes with the addition of NaCas. This shift was confirmed by the significantly higher $D[4,3]$ values observed for the powder particles with increasing casein content (the grey points in Fig. 2C). As with the BI, a maximum for the $D[4,3]$ value was observed in powders containing around 40% casein. This increase in $D[4,3]$ was probably due to higher resistance of powders with higher NaCas contents (or lower lactose contents) to crushing of the freeze-dried powder during preparation for DH. However, it is unclear why this increase in $D[4,3]$ was not linear with increasing casein content. This change in size of the powder flakes may be influenced by the freeze-drying process. Firstly, freeze-drying begins with freezing of the solution leading to separation of the protein solution into, first, liquid water in the form of ice

crystals and, second, a concentrated solution of proteins and solutes. Ice is subsequently removed during the sublimation step, together with the water adsorbed onto the solutes (Franks, 1998). Concentrated proteins and solutes may then interact, be denatured, and either precipitate in the crystalline state or stay as an amorphous solid that can also proceed to glass transition at low temperatures. We assumed that the structure of the freeze-dried cake before the crushing step was made up of pores the size of ice crystals embedded in the protein amorphous concentrate, and that the crushing would lead to breakage preferentially through these pores. Thus, the structure of the cake would be expected to have consequences on the size of the powder flakes: a larger crystal size distribution would lead to a cake with larger pores, a denser amorphous protein phase, and to smaller flakes than would be observed with smaller crystals. However, this hypothesis cannot be confirmed as we do not know the sizes of the crystals in the different frozen mixtures. Secondly, although the increase in size of the powder flakes appeared to correlate with the small decrease in lactose content resulting from substitution of WP by NaCas, reduced amounts of lactose are very unlikely to lead to an increase in powder flake size as decreasing the lactose content has been shown previously to decrease flake sizes (Schong & Famelart, 2019). Thirdly, protein powders are more rigid and brittle at temperatures below the glass transition temperature (T_g) and softer and more viscous-like at temperatures above the T_g . The T_g values of NaCas, Casmic and WP at an $a_w \sim 0.3$ are 61, 72 and 84 °C, respectively (Schuck, Jeantet, & Dolivet, 2012). The lower the T_g of the powder, the closer the powder would be to the transition towards a softer material, and the softer the material would be. Thus, we would expect Cas18 to be harder than Cas99, but the observed flake sizes contradict this explanation. Furthermore, differences in behavior between WP and micellar casein, with relevant consequences on powder morphology, have been reported previously during spray-drying (Sadek et al., 2014). Powder particles of micellar casein are wrung, and invaginated, whereas those of WP are totally spherical, and present a large vacuole of occluded air and a smooth surface. These differences could be ascribed to the fact that when proteins are concentrated to above a critical concentration during droplet drying, casein or caseinate

undergo a sol-gel transition (Bouchoux et al., 2009) and globular proteins undergo a glass transition (Brownsey, Noel, Parker, & Ring, 2003; Parker, Noel, Brownsey, Laos, & Ring, 2005), leading to development of solid-like properties. Storage moduli of 200-300 Pa for casein gels and > 10000 Pa for β -Lg and bovine serum albumin gels have been reported at concentrations of 100-200 g/kg for casein and > 400 g/kg for globular proteins (Bouchoux et al., 2009; Brownsey et al., 2003; Parker et al., 2005). Thus, the differences in powder morphology and size may have been caused by differences in the physicochemical properties of the proteins, such as their transition concentration or gel firmness. However, casein gels formed by concentration are softer than WP gels, a finding which again is contradictory to our observation that flake size increases with increasing casein content. In conclusion, the mechanism underlying the changes in flake size with altered composition of the mixtures remains unclear. The observed changes in flake size were small, with the size of powder flakes ranging between 5 and 500 μ m and the D[4,3] values ranging between 60 and 100 μ m for all powders; however, any changes in flake size or structure could have major consequences on the formation of PMs.

Light micrographs of powder particles from samples of Cas18, Cas58 and Cas99 after DH, and from Casmic before and after DH, are shown in Fig. 3. The powder particles appear as flakes, as expected for freeze-dried powders. Although microscopy could not be used to provide accurate evaluations of the size distribution of the flakes, because of the very small number of particles observed in each sample and the risk that these small samples were unrepresentative of the bulk, we did not notice any significant size differences between the flakes of powders with different NaCas contents. The Cas99 powder flakes appeared slightly denser and more shrunken than flakes from powders with lower NaCas contents. In addition, the Casmic powder flakes appeared much more shrunken, less translucent, and much smaller than flakes from the other powders. It was of interest to note that the aspect of the Casmic flakes, and indeed that of all the other powders studied (data not shown), was not modified by the DH treatment. The size of the flakes from powders with WP and NaCas

appeared to be around 5–20 μm , whereas those from the Casmic powder were $< 10 \mu\text{m}$. These estimates of flake size made from microscopic evaluations were smaller than the values obtained using the Qicpic method. We consider that the Qicpic method gave the most reliable estimation of flake size because using this method more than 10^6 particles were characterized during one measurement.

3.2. Protein microparticle characterization

Photographs of S0 suspensions of dry-heated powders were taken immediately (d0, not shown) and 5 d after their rehydration and quiescent storage (see the supplementary information and Fig. S1). The d0 suspensions of dry-heated powders appeared cloudy, due to the presence of PMs that sedimented after storage. These suspensions were yellow for the Cas18-Cas99 samples, and yellow-red for the Casmic sample. In contrast, the suspensions of powders before DH were translucent (not shown). Similar volumes of sediment appeared to be present in the Cas18 to Cas38 samples, but the volume of sediment seemed to decrease slightly in suspensions from powders with above 38% casein. A far smaller volume and browner sediment was observed in the Casmic sample. These findings show that DH of the powders for 36 h led to the formation of PMs that sedimented with time. Evaluation of the height of the sediment on the photograph revealed that these sedimented particles occupied a rather large volume: although the suspensions contained only 1% dry-heated powder, the sedimented particles occupied between 10% (Casmic) and 24-17% (WP and NaCas) of the total volume.

The size distribution of the PMs varied between 4 and 1000 μm for the Cas18 suspension and seemed to shift towards larger values with the addition of increasing amounts of NaCas (as shown in Fig. 2B). $D[4,3]$ values increased as the NaCas content increased from 18% to around 40% and then decreased for samples with over 40% NaCas (Fig.2C). The size of these PMs was 2-4 fold larger than that of the powder flakes, with the Cas34-Cas44 samples having the largest PM sizes. The SR was around four for the Cas34 sample and the lowest

SR value, of around two, was obtained for Cas99. Swelling of the dry-heated particles when placed in the aqueous phase, leading to larger sizes in suspension than those observed in the powder, has already been reported in previous studies (Schong & Famelart, 2018): the diameter of particles obtained by DH of spray-dried powders has been shown to increase by a factor of four when placed in water. This swelling was probably due to repulsion between negative charges on proteins inside the PMs. An example of this swelling is shown for a flake from the Cas99 sample and occurred within around 20 s under the conditions used in the current study (see supplementary Fig. S2): the dimensions of the flake increased by a factor of around two, in accordance with the calculated SR of powder flakes from this sample (SR \approx 2). Our previous study indicated that the degree of swelling was related to the number of crosslinks between proteins inside the PM: swelling was reduced after prolonged DH times and in powders with higher lactose contents, i.e. under conditions where the number of crosslinks increased (Schong & Famelart, 2019). However, the degree of swelling may also be related to the size of the powder flakes: in the current study swelling was maximal in samples with 34-44% casein. It should be noted that the maximum BI was also observed in samples with around 40% casein.

The size of PMs from the Casmic suspension varied between around 2 and 1000 μm and their $D[4,3]$ was close to the $D[4,3]$ of the powder flakes. In contrast to the WP-NaCas flakes, the Casmic PMs underwent only minimal swelling when placed in water, and some of the PMs appeared to be smaller than the powder particles (1-10 μm ; Fig. 2B). As these small PMs swelled when placed in suspension, they may have been present in the powder, but were smaller than the minimum size detected by the QICPIC method ($\sim 1 \mu\text{m}$). Although PMs were formed by DH of the Casmic powder, the suspended particles appeared to be smaller than the PMs observed in the other powder samples analyzed in this study. The small size of the Casmic PMs probably resulted from a high degree of crosslinking associated with very intense Maillard reactions promoted by the higher lactose content of this sample.

In conclusion, dry-heated powders with WP and NaCas swelled by a factor of 2-4, whereas no swelling was observed for the Casmic powder. CLSM micrographs showing the sizes of the PMs in the samples are shown in Fig. 4. No particles were visible in the powder suspensions before DH (data not shown). Angular PMs, with shapes similar to those observed for the powder flakes, were formed by DH of all the powders. The largest PMs were observed for powders with between 34 and 44% casein. PMs from the Casmic powder appeared to be much smaller than the those formed from powders containing WP and NaCas. These findings are therefore in accordance with the size measurements obtained using the Mastersizer method (Fig. 2).

The yields of conversion of the powder into PMs (DY and WY) and the WC of the PMs are shown in Fig. 5. The DY value of 0.6 g/g for the WP sample without added casein was the same as that reported in our previous study (0.6-0.7 g/g; Famelart et al., 2018; Schong & Famelart, 2019). The DY of PMs produced from WP and NaCas powders increased slightly with increasing NaCas content (+31% between Cas18 and Cas99). The fact that the DY increased with the caseinate content confirmed that caseinate formed part of the PMs, with the caseinate molecules being crosslinked by DH at pH 9.5. If the caseinate had not formed part of the PMs, we would have expected to see a decrease in DY with increasing NaCas content. It seems that the increase in DY with casein content (i.e. the slope of the DY-casein content curve) was most rapid between 18% and 40% casein, and slower at higher casein contents from 40 to 99%. The amount of water entrapped in the PMs (WC) from the powder without added casein was 28 g/g. This value is in accordance with the WC values of 25-30 g/g found for the same freeze-dried powder in our previous studies (Famelart et al., 2018; Schong & Famelart, 2019), but is lower than the value of 49 g/g found for the spray-dried powder after the same DH treatment (Schong & Famelart, 2018). WC decreased with the addition of NaCas, but this decrease was irregular: the decrease was minimal from 18 to 34% and from 50 to 99% casein, whereas it was maximal between 34 and 58% casein. The WY from the powder without added casein was 18 g/g, compared to 18-20 g/g in previous

studies (Famelart et al., 2018; Schong & Famelart, 2018, 2019). The WY increased between 18 and 34-38% casein and then declined for powders with above 38% casein before becoming almost constant between 58 and 99% casein. In conclusion, the yields and WC values were slightly modified by the substitution of WP for NaCas. Replacing WP by NaCas promoted the formation of more PMs, but with less water entrapped in their structures. These small differences could be due to: i) changes in the size or structure of the crushed powders, with flakes produced from the Cas18 powder being smaller (Fig.2C) and those produced from Cas99 being denser and more shrunken (Fig.3); ii) differences in the amount of unfolded protein, such as casein; iii) differences in the extent of crosslinking during DH, associated with differences in lactose content. Indeed, lactose has been shown to promote the rapid formation of PMs (Schong & Famelart, 2019). However, lactose content alone cannot explain the changes observed with increasing casein content, as maximal values for the BI, particle swelling and WY were obtained for powders with around 40% casein. As discussed above, the structure of the cake would be expected to change with the size and number of ice crystals formed during freezing of the protein solution, the amount of lactose or any other solute, and with the physicochemical properties of the proteins, such as their Tg and transition concentration and the gel firmness of their concentrated solutions. As the density of the protein cake, together with the extent of the crosslinking and the protein-protein and protein-water interactions responsible for swelling, define the amount of water in the particles in suspension (i.e. the WC), it would be necessary to characterise all of these parameters to be able to fully explain the variation in properties of the PMs produced by DH.

The fact that the maximum BI, powder flake and PM D[4,3] and WY values were all observed at around 40% casein could also be explained by a specific interaction occurring at pH 9.5 between casein and WP at this ratio, with consequences on protein behaviour. This hypothesis needs to be confirmed by analysis of the proteins in solution at pH 9.5 before drying and after drying and DH.

The Casmic powder also formed large PMs during DH, with a higher DY than the WP and NaCas powders (Fig. 5). This higher DY indicates that more extensive crosslinking leads to retention of the micellar casein inside the powder particles during the DH and that fewer proteins were present as small aggregates or monomers in the soluble phase. These PMs entrapped much less water and displayed lower WC and WY values than those observed for the WP and NaCas powders (Fig. 5). The limited ability of the Casmic PMs to retain water is related to the fact that the D[4,3] values of the powder and PMs were very similar, thus these PMs did not swell when dissolved in water. Moreover, we have already shown that an increase in the DY of WP PMs correlates with a lower WC (Schong & Famelart, 2019).

3.3. Denaturation of whey proteins and composition of protein microparticles

For powders made of WP and NaCas, the level of WP denaturation before DH and after 36 h of DH was almost constant, regardless of the NaCas content (Table 1). Before DH, 70% of the WPs were still native, meaning that 30% were already denatured before DH. We have already shown that adjusting the pH of the WP solution to 9.5, followed by drying and a_w adjustment of the powder at 20 °C for 2 weeks, leads to WP denaturation: similar values of 50-60% (Famelart et al., 2018) and 70-80% (Schong & Famelart, 2018, 2019) residual NWP were obtained before DH in previous studies. β -Lg was shown previously to be the main protein impacted by the pH adjustment: the native α -lac and bovine serum albumin (BSA) contents before DH were 90%, whereas that of β -Lg was only 50% (Famelart et al., 2018). Indeed, alkaline-induced changes in WPs have been reported at pHs above 9, including irreversible unfolding and polymerization of proteins (Monahan, German, & Kinsella, 1995; Partanen et al., 2011; Taulier & Chalikian, 2001).

The NWP content decreased to 10-20% after 36 h of DH, and both β -Lg and α -lac were denatured. These values are similar to those obtained in previous studies (Famelart et al., 2018; Schong & Famelart, 2018, 2019). BSA has also been shown to be denatured by DH (Famelart et al., 2018), thus we can state that most WPs are denatured by the DH treatment

at alkaline pH values. Finally, increasing the NaCas content did not have any effect on WP denaturation during the DH process. It was not possible to analyze the levels of NWP in samples from the Cas99 powder containing only 1% WP.

For the Casmic powder, the residual NWP content before DH was 100% (Table 1). One hypothesis for the lack of denaturation of the WPs in the Casmic powder is that these WP were less sensitive to the alkaline pH. Although the Casmic sample contained only 7% WP, the degree of denaturation after 36 h of DH was similar to that detected in the WP and NaCas powders, at around 90%. These findings show that WPs from the Casmic powder were also sensitive to DH at an alkaline pH.

4. Conclusions

Our study shows that DH of a powder made of WP and NaCas conditioned at pH 9.5 led to the production of large and highly hydrated PMs. Substitution of WP by NaCas led to small changes in the formation of PMs and in their properties. The yield of conversion of protein into PMs tended to increase, whereas the amount of water entrapped in these PMs tended to decrease with the substitution of WP by casein. However, these changes were limited and not linearly dependent on the casein content. The size of the PMs was maximal in powders with around 40% casein. We showed PM formation also occurs in micellar casein-enriched milk protein powders. That fact that casein has no native structure but was still able to form PMs, throws doubt on the hypothesis that the role of the alkaline pH conditions used in the process was to unfold proteins, as suggested in Schong & Famelart (2019). Furthermore, these data suggest that it is possible to produce PMs by this process for a range of powders, including whey isolate or concentrate or a milk concentrate, as casein has only a limited effect on the yield and properties of the particles. Further studies are required to characterize the effects of this process on spray-dried powders.

456 **Acknowledgments**

457 We are grateful to the Regional councils of Brittany (grant N°13008651) and Pays de la
458 Loire (grant n°2014-07081) for financial support and INRA for scientific coordination (J.
459 Leonil) through the interregional project PROFIL, managed by the BBA industrial association,
460 and to P. Hamon and F. Rousseau for their technical help with HPLC analyses and particle
461 size analyses.

References

- Bouchoux, A., Debbou, B., Gesan-Guizieu, G., Famelart, M. H., Doublier, J. L., & Cabane, B. (2009). Rheology and phase behavior of dense casein micelle dispersions. *Journal of Chemical Physics*, 131(16).
- Brownsey, G. J., Noel, T. R., Parker, R., & Ring, S. G. (2003). The Glass Transition Behavior of the Globular Protein Bovine Serum Albumin. *Biophysical Journal*, 85(6), 3943–3950.
- Dickinson, E. (2015). Microgels — An alternative colloidal ingredient for stabilization of food emulsions. *Trends in Food Science & Technology*, 43(2), 178–188.
- Dissanayake, M., Liyanaarachchi, S., & Vasiljevic, T. (2012). Functional properties of whey proteins microparticulated at low pH. *Journal of Dairy Science*, 95(4), 1667–1679.
- Famelart, M.-H., Schong, E., & Croguennec, T. (2018). Dry heating a freeze-dried whey protein powder: Formation of microparticles at pH 9.5. *Journal of Food Engineering*, 224, 112–120.
- Fenaille, F., Morgan, F., Parisod, V., Tabet, J.-C., & Guy, P. A. (2003). Solid-state glycation of β -lactoglobulin monitored by electrospray ionisation mass spectrometry and gel electrophoresis techniques. *Rapid Communications in Mass Spectrometry*, 17(13), 1483–1492.
- Franks, F. (1998). Freeze-drying of bioproducts: putting principles into practice. *European Journal of Pharmaceutics and Biopharmaceutics*, 45(3), 221–229.
- Friedman, M. (1996). Food Browning and Its Prevention: An Overview. *Journal of Agricultural and Food Chemistry*, 44(3), 631–653.
- Gaucheron, F. (2003). *Minéraux et Produits Laitiers*. Tec & Doc.
- Gerrard, J. A., Meade, S. J., Miller, A. G., Brown, P. K., Yasir, S. B. M., Sutton, K. H., & Newberry, M. P. (2005). Protein Cross-Linking in Food. *Annals of the New York Academy of Sciences*, 1043(1), 97–103.
- Gerrard, J. A. (2002). Protein–protein crosslinking in food: methods, consequences, applications. *Trends in Food Science & Technology*, 13(12), 391–399.

490 Gulzar, M., Bouhallab, S., Jeantet, R., Schuck, P., & Croguennec, T. (2011). Influence of pH
 491 on the dry heat-induced denaturation/aggregation of whey proteins. *Food Chemistry*,
 492 129(1), 110–116.

493 Hiller, B., & Lorenzen, P. C. (2010). Functional properties of milk proteins as affected by
 494 Maillard reaction induced oligomerisation. *Food Research International*, 43(4), 1155–
 495 1166.

496 International Dairy Federation. (2004). Milk - Determination of casein-nitrogen content - Part
 497 2: Direct method. *International Standard*, 29.2.

498 International Dairy Federation. (2010). Milk, cream and evaporated milk - Determination of
 499 total solids content (Reference method). *International Standard*, 21B.

500 International Dairy Federation. (2014). Milk and dairy products - Determination of nitrogen
 501 content Part 1: Kjeldahl principle and crude protein. *International Standard*, 20–1.

502 International Dairy Federation. (2016). Milk and dairy products - Determination of nitrogen
 503 content Part 4: Determination of protein and non-protein nitrogen content and true
 504 protein content calculation (Reference method). *International Standard*, 20–4.

505 Inthavong, W., Kharlamova, A., Chassenieux, C., & Nicolai, T. (2016). Structure and flow of
 506 dense suspensions of protein fractal aggregates in comparison with microgels. *Soft
 507 Matter*, 12(10), 2785–2793.

508 Kharlamova, A., Chassenieux, C., & Nicolai, T. (2018). Acid-induced gelation of whey protein
 509 aggregates: Kinetics, gel structure and rheological properties. *Food Hydrocolloids*,
 510 81, 263–272.

511 Lan, X., Liu, P., Xia, S., Jia, C., Mukunzi, D., Zhang, X., ... Xiao, Z. (2010). Temperature
 512 effect on the non-volatile compounds of Maillard reaction products derived from
 513 xylose–soybean peptide system: Further insights into thermal degradation and cross-
 514 linking. *Food Chemistry*, 120(4), 967–972.

515 Loveday, S. M., Su, J., Rao, M. A., Anema, S. G., & Singh, H. (2012). Whey Protein
 516 Nanofibrils: The Environment–Morphology–Functionality Relationship in

517 Lyophilization, Rehydration, and Seeding. *Journal of Agricultural and Food*
 518 *Chemistry*, 60(20), 5229–5236.

519 Martins, S. I. F. S., & Van Boekel, M. A. J. S. (2005). Kinetics of the glucose/glycine Maillard
 520 reaction pathways: influences of pH and reactant initial concentrations. *Food*
 521 *Chemistry*, 92(3), 437–448. <https://doi.org/10.1016/j.foodchem.2004.08.013>

522 Maskan, M. (2001). Kinetics of colour change of kiwifruits during hot air and microwave
 523 drying. *Journal of Food Engineering*, 48(2), 169–175.

524 Miller, A. G., Meade, S. J., & Gerrard, J. A. (2003). New insights into protein crosslinking via
 525 the Maillard reaction: structural requirements, the effect on enzyme function, and
 526 predicted efficacy of crosslinking inhibitors as anti-ageing therapeutics. *Bioorganic &*
 527 *Medicinal Chemistry*, 11(6), 843–852.

528 Monahan, F. J., German, J. B., & Kinsella, J. E. (1995). Effect of pH and temperature on
 529 protein unfolding and thiol/disulfide interchange reactions during heat-induced
 530 gelation of whey proteins. *Journal of Agricultural and Food Chemistry*, 43(1), 46–52.

531 Nicolai, T., & Durand, D. (2013). Controlled food protein aggregation for new functionality.
 532 *Current Opinion in Colloid & Interface Science*, 18(4), 249–256.

533 Nicolai, Taco, Britten, M., & Schmitt, C. (2011). β -Lactoglobulin and WPI aggregates:
 534 Formation, structure and applications. *Food Hydrocolloids*, 25(8), 1945–1962.

535 O'Mahony, J. A., Drapala, K. P., Mulcahy, E. M., & Mulvihill, D. M. (2017). Controlled
 536 glycation of milk proteins and peptides: Functional properties. *International Dairy*
 537 *Journal*, 67, 16–34.

538 Parker, R., Noel, T. R., Brownsey, G. J., Laos, K., & Ring, S. G. (2005). The nonequilibrium
 539 phase and glass transition behavior of β -Lactoglobulin. *Biophysical Journal*, 89(2),
 540 1227–1236.

541 Partanen, R., Torkkeli, M., Hellman, M., Permi, P., Serimaa, R., Buchert, J., & Mattinen, M.-L.
 542 (2011). Loosening of globular structure under alkaline pH affects accessibility of β -
 543 lactoglobulin to tyrosinase-induced oxidation and subsequent cross-linking. *Enzyme*
 544 *and Microbial Technology*, 49(2), 131–138.

545 Sadek, C., Li, H., Schuck, P., Fallourd, Y., Pradeau, N., Le Floch-Fouéré, C., & Jeantet, R.
 546 (2014). To what extent do whey and casein micelle proteins influence the morphology
 547 and properties of the resulting powder? *Drying Technology*, 32(13), 1540–1551.

548 Schmitt, C., Bovay, C., & Frossard, P. (2005). Kinetics of formation and functional properties
 549 of conjugates prepared by dry-state incubation of β -Lactoglobulin/acacia gum
 550 electrostatic complexes. *Journal of Agricultural and Food Chemistry*, 53(23), 9089–
 551 9099.

552 Schong, E., & Famelart, M.-H. (2017). Dry heating of whey proteins. *Food Research*
 553 *International*, 100(Pt 2), 31–44.

554 Schong, E., & Famelart, M.-H. (2018). Dry heating of whey proteins leads to formation of
 555 microspheres with useful functional properties. *Food Research International*, 113,
 556 210–220.

557 Schong, E., & Famelart, M.-H. (2019). Influence of lactose on the formation of whey protein
 558 microparticles obtained by dry heating at alkaline pH. *Food Hydrocolloids*, 87, 477–
 559 486.

560 Schuck, P., Jeantet, R., & Dolivet, A. (2012). *Analytical methods for food and dairy powders*.
 561 John Wiley & Sons.

562 Taulier, N., & Chalikian, T. V. (2001). Characterization of pH-induced transitions of β -
 563 lactoglobulin: ultrasonic, densimetric, and spectroscopic studies. *Journal of Molecular*
 564 *Biology*, 314(4), 873–889.

565 Wei, Q., Liu, T., & Sun, D.-W. (2018). Advanced glycation end-products (AGEs) in foods and
 566 their detecting techniques and methods: A review. *Trends in Food Science &*
 567 *Technology*, 82, 32–45.

568 Zamora, R., & Hidalgo, F. J. (2005). Coordinate contribution of lipid oxidation and Maillard
 569 reaction to the nonenzymatic food browning. *Critical Reviews in Food Science and*
 570 *Nutrition*, 45(1), 49–59.

571 Zhang, Z., Arrighi, V., Campbell, L., Lonchamp, J., & Euston, S. R. (2016). Properties of
572 partially denatured whey protein products 2: Solution flow properties. *Food*
573 *Hydrocolloids*, 56, 218–226.

574

575 **Table 1**

576 Native residual whey protein (NWP) levels in S0 suspensions of powders before and after 36
 577 h of dry heating (DH) at 100 °C. Data are expressed as the % of total WP in the S0
 578 suspension.

Casein content (% w/w)	NWP	
	Before DH	After DH
18	75.4±5.1 ^a	14.6±2.1 ^a
34	77.7±7.0 ^a	14.4±2.7 ^a
38	77.2±4.3 ^a	13.9±1.2 ^a
44	71.9±0.5 ^a	14.3±2.4 ^a
58	78.6±3.7 ^a	10.9±1.6 ^a
93*	102.7±2.9 _b	8.3±0.6 ^b

579 * Micellar casein-enriched milk protein powder sample, all other samples are mixtures of
 580 sodium caseinate (NaCas) and whey protein (WP) powder.

581 ^{a,b} For each column, values marked with different letters showed a statistically significant
 582 difference in NWP content (P < 0.05).

583 **Figure captions**

584 **Fig. 1.** The browning index (BI) of powders before (grey) and after 36 h of DH at 100 °C
585 (black) as a function of the percentage casein content (w/w). The powders analyzed
586 contained either whey proteins with increasing amounts of sodium caseinate (circles) or
587 micellar casein-enriched milk proteins (crosses). Plotted lines illustrate the trend in BI with
588 increasing casein content.

589 **Fig. 2.** The granular size distribution of A) freeze-dried powder particles before dry heating
590 (DH) and B) protein microparticles in S0 suspensions after DH of the powders. Colors from
591 black to green represent powders with increasing amounts (w/w) of sodium caseinate
592 (NaCas), ranging from Cas18 with a mixture of whey proteins and 18% NaCas to Cas99 with
593 a mixture of whey protein and 99% NaCas. The red curve represents the particle size
594 distribution for the micellar casein-enriched powder (Casmic). The distributions shown are
595 representative of data obtained from two samples of each S0 suspension measured in
596 triplicate; C) The mean volume diameter (D[4,3]) of powder particles (grey) and of protein
597 microparticles (black) as a function of casein content for powders containing either whey
598 proteins with increasing amounts of sodium caseinate (circles) or micellar casein-enriched
599 milk proteins (crosses). For each curve, no statistical differences were observed between
600 samples for data points marked with the same letter ($P < 0.05$).

601 **Fig. 3.** Light micrographs of particles from powders containing whey proteins with increasing
602 amounts (w/w) of sodium caseinate (NaCas) ranging from 18% (Cas18) to 99% (Cas99) after
603 36 h of dry heating (36h DH), and of the particles from the powder containing micellar casein-
604 enriched proteins (Casmic) before DH (0h DH) and after 36 h of DH (36h DH). The
605 micrographs shown are representative of the 10 images recorded for each sample.

606 **Fig. 4** Confocal laser scanning micrographs of S0 suspensions of powders dry-heated for 36
607 h. Suspensions were prepared from: A) powders of whey proteins with increasing amounts

608 (w/w) of sodium caseinate (NaCas), ranging from 18% (Cas18) to 99% (Cas99); and B)
609 Casmic powder of micellar casein-enriched proteins. Bars = 200 μ m. The two micrographs
610 shown for each powder are representative of the 10 images recorded for each sample.

611 **Fig. 5.** Dry yield (DY) of conversion of powder dry matter into microparticles, water content
612 (WC) of the microparticles and wet yield (WY) measured as the weight of pellets from S0
613 suspensions as a function of the percentage casein content in % (w/w). The dry-heated
614 powders in the suspensions contained either whey proteins with increasing amounts of
615 sodium caseinate (circles) or micellar casein-enriched milk proteins (crosses).

Fig 1

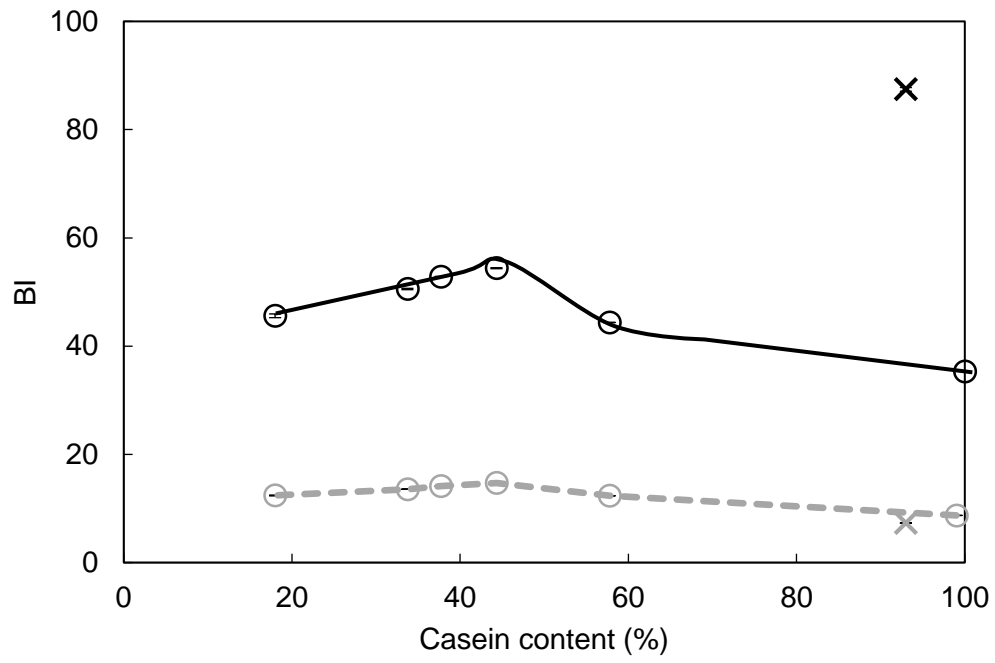


Fig. 2.

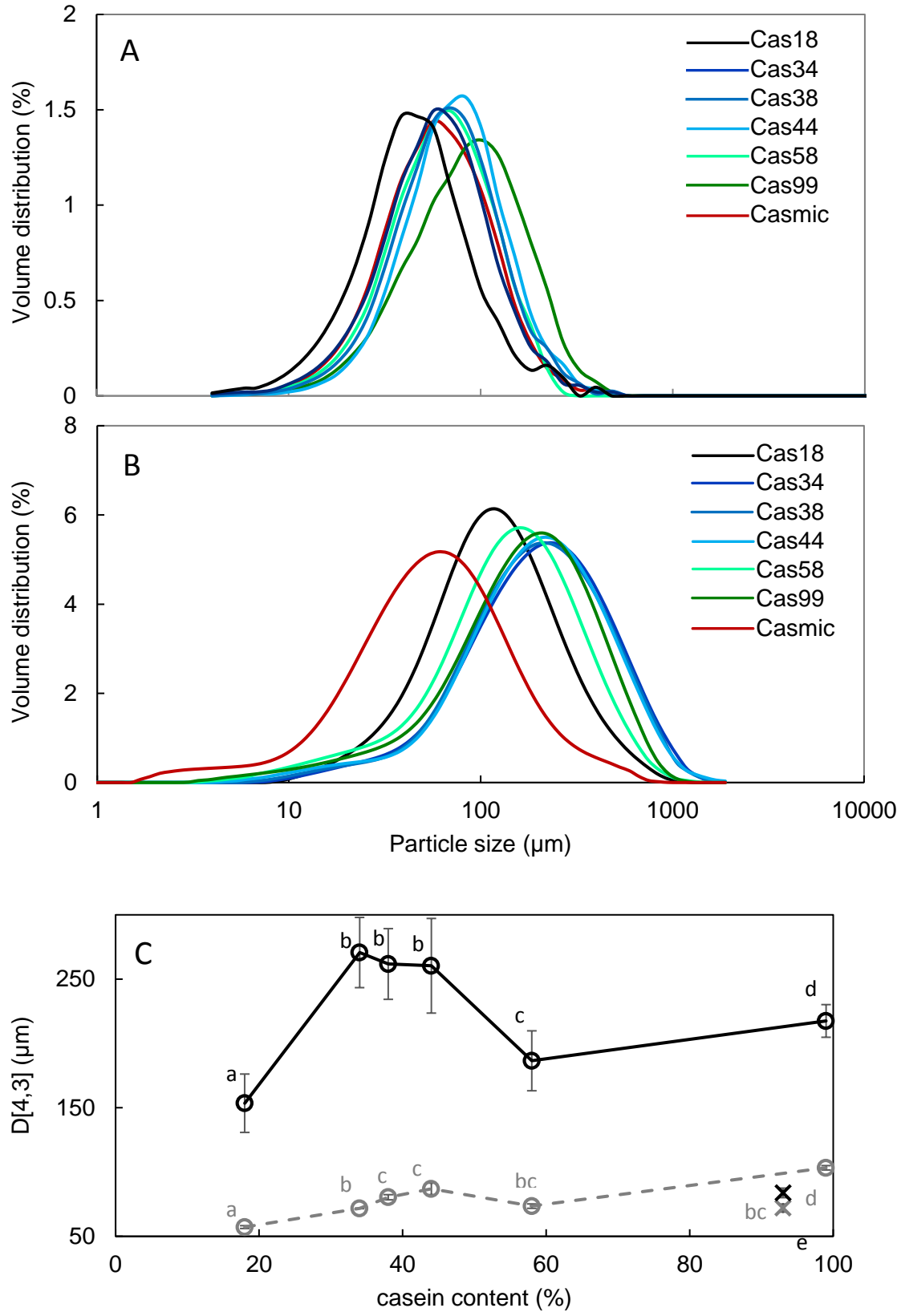
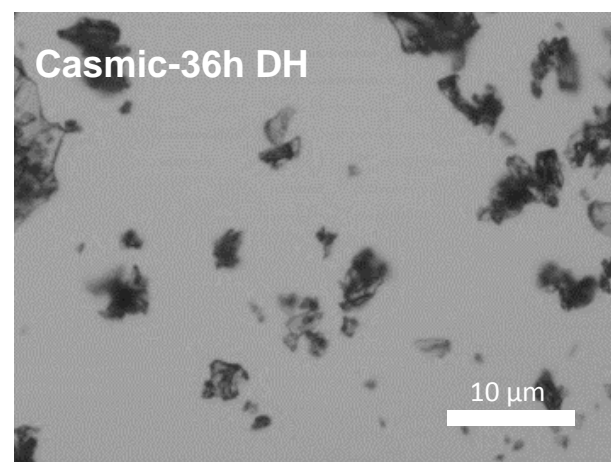
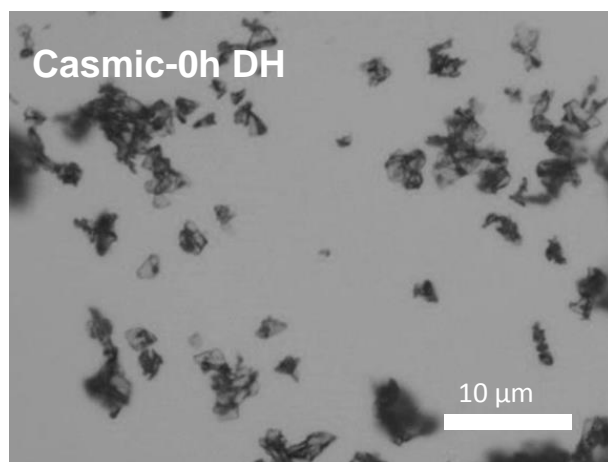
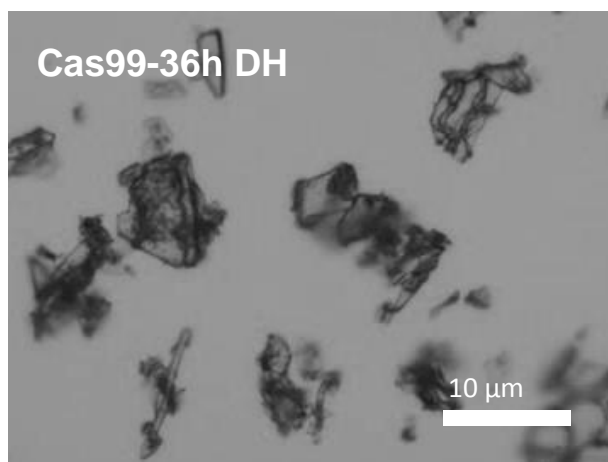
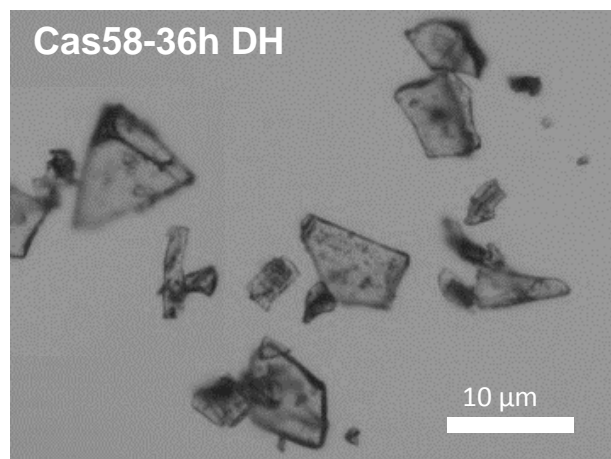
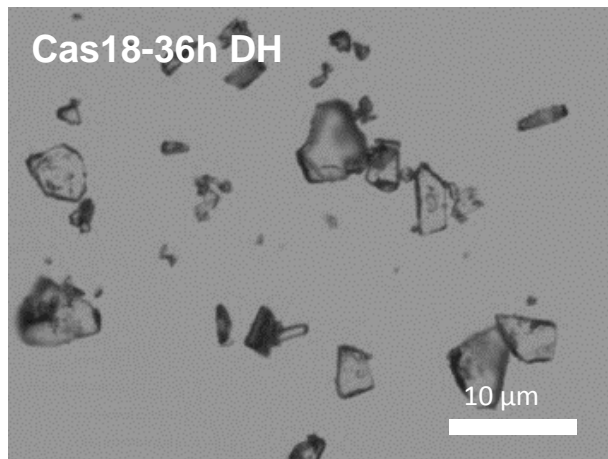


Fig. 3



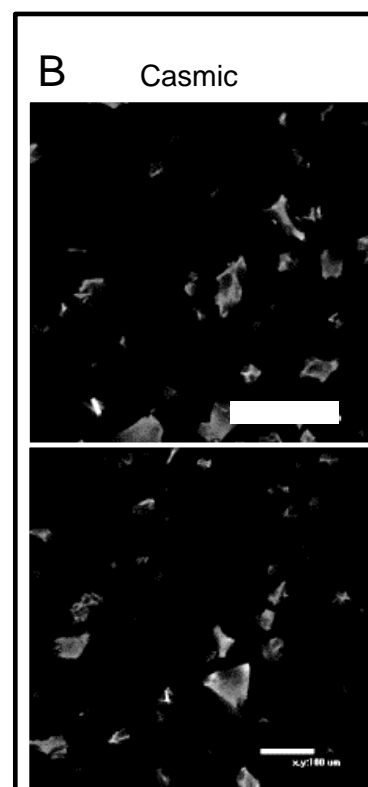
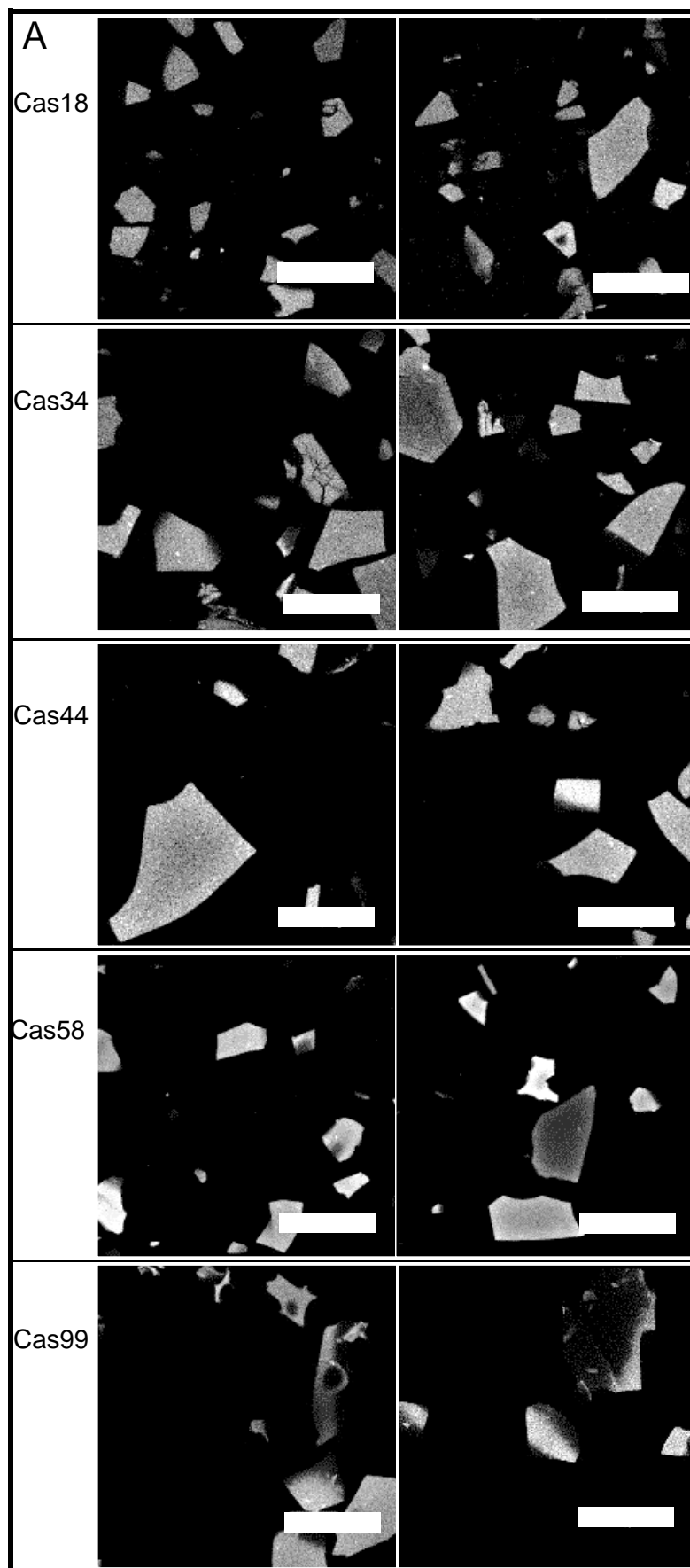


Fig. 5

