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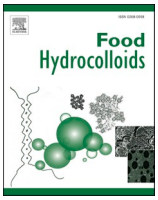

Structural and compositional characterization of Ca- and β-casein enriched casein micelles

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ABSTRACT

There is an increased interest in understanding the potential of modifying casein micelles composition, or even creating them *de novo*. In this study, soluble casein fractions were created by dissociation from native casein micelles, and their properties were studied in isolation, and after their addition to native casein micelles. The structure of these enriched casein micelles was investigated by small-angle X-ray scattering (SAXS) to evaluate if the enrichment caused changes in their internal structure. Acidification of native casein micelles from pH 6.8 to 5.6 caused colloidal calcium phosphate dissociation, and SAXS data showed a decrease in intensity at $q = 0.08$ Å^{-1}, where q is the modulus of the scattering vector. Additionally, the acidification of native casein micelles (to pH 6.0) and incubation at 4 °C caused a decrease in scattering intensity at the intermediate q region (0.01–0.02 Å^{-1}), signifying a loss of heterogeneity among the intermediate structure elements in the casein micelle. This change was caused by micellar dissolution of calcium phosphate and the release of β-casein at 4 °C. After the resuspension of casein micelles with a β-casein enriched serum, the absence of soluble β-casein suggested that most β-casein was re-associated in the colloidal fraction. These samples showed no changes in the structural features probed by SAXS. The work brings new evidence that, while a small extent of dissociation may affect the structure of the casein micelles, the enrichment with β-casein results in similar structures as those of the native micelle.

1. Introduction

Bovine skim milk is a heterogeneous mixture of water, proteins, salts, sugars, and residual lipids. The proteins present in milk can be subdivided into two groups: The whey proteins (WP) and caseins, constituting 20% and 80% of the total protein pool, respectively. The major whey proteins are constituted by β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), and the casein proteins are constituted by κ-, α_{S1}-, α_{S2}- and β-casein. Multiple genetic variants exist among the proteins, on top of a number of variants produced by post-translational modifications, such as glycosylations and phosphorylations. The minerals of milk contain cations (Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}) and anions (inorganic PO_4^{-}, citrate^{-}, and Cl^{-}).

In native skim milk, the components partition between the soluble (serum) and colloidal (also more commonly referred to as micellar) phases. At a pH ranging from 6.6 to 6.8 and at room temperature, 95% of the casein proteins are arranged in colloidal structures called casein micelles, which also contain on a dry matter basis, approximately 6% minerals, mainly Ca and PO_4 (Fox, Uniacke-Lowe, Mcsweeney, & O'Mahony, 2015). The rest of the minerals, whey proteins, and lactose are present in the serum phase. The casein proteins are rich in Proline amino acids, providing open structures prone to change with environmental conditions (Holt & Sawyer, 1993). In addition, they contain localized phosphoserine residues and sequences of predominantly hydrophobic or hydrophilic amino acids, promoting self-associations through non-covalent interactions. Two thirds of all Ca and half of the inorganic PO_4 (P_i) present in milk is in the micellar phase (Gaucheron, 2005). The Ca and P_i are associated with the casein micelle through local, charged moieties of the casein proteins. Especially the Ser-linked phosphorylation centers on α_{S1}-, α_{S2}-, and β-caseins bind $CaPO_4$ salts, thereby forming colloidal $CaPO_4$ (CCP) nanoclusters. The κ-casein is mostly located at the surface of the micelle due to the hydrophilic nature of its C-terminus forming a polyelectrolyte 'hairy' layer (Dalgleish, Horne, & Law, 1989; Holt & Horne, 1996; Marchin, Putaux, Pignon, &

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Leonil, 2007). This outer layer, containing glycosylated κ-casein, stabilizes the colloidal casein micelles preventing them from aggregating with one another, because of electrostatic and steric stabilization (De Kruif & Zhulina, 1996). The casein micelles can be destabilized by the specific hydrolysis of κ-casein by the enzyme chymosin, resulting in the prompt aggregation of the casein micelles (Dalgleish & Corredig, 2012; De Kruif & Zhulina, 1996).

With changes in environmental conditions such as temperature and pH, the components present in the micellar and soluble phases change. If milk is stored at a temperature less than about 10 °C, the hydrophobic interactions within the micelles are weakened and β-casein dissociates from the micelles, because of the hydrophobic nature of a large portion of its peptide chain (Creamer, Berry, & Mills, 1977; Yazdi, Corredig, & Dalgleish, 2014). If some of the negative charges are neutralized by a pH decrease, a portion of the colloidal $CaPO_4$ solubilizes resulting in an increase in the concentration of $CaPO_4$ in the soluble phase (Gaucheron, 2005). Precipitation of caseins will occur at their isoelectric point, around pH 4.6.

With modern technologies, it has become increasingly possible to produce milk fractions with different compositions. Temperature and pH can affect the dissociation of caseins from the micelles (Dalgleish & Law, 1988, 1989; Møller, Nielsen, & Corredig, 2023; Post, Arnold, Weiss, & Hinrichs, 2012), and using membrane filtration, it is then possible to demineralize, concentrate or fractionate the colloidal fraction, collecting, depending on the membrane size and use, the soluble material produced, in the permeate fraction. This process may result in the creation of new functional ingredients. It is therefore becoming important to have tools able to evaluate potential changes in the structure of the casein micelles during micellar dissociation or enrichment.

Of particular interest is the dissociation of β−casein and its potential to be isolated and recombined in new ingredients. When microfiltration of skim milk is performed at 4 °C, a stream rich in β-casein is obtained due to weakening of hydrophobic interactions causing its solubilization (Creamer et al., 1977) allowing its migration across the membrane (Hekken & Holsinger, 2000; McCarthy, Wijayanti, Crowley, O'Mahony, & Fenelon, 2017). This protein has proven to be of interest, because of its presence in a high ratio compared to the other caseins in human milk. Research that has been centered around nutritional supplementation has then focused on the enrichment of β-casein streams and the digestibility of these new ingredients in *in vitro* infant and adult digestion models (D. Liu et al., 2019; D. S. Liu et al., 2016; McCarthy et al., 2017). Therefore, it is of great interest to further understand how to obtain casein micelles with different composition, as for example, micelles enriched in β-casein, and how the processes of assembly and disassembly affect their structure, as these may lead to changes in functionality.

As technologies may create new ingredients with casein assemblies of different compositions, partly dissociating or enriching native micelles, more research is needed to understand the potential of their recombination/enrichment or the added value resulting from their functional properties. The present research aimed to create soluble fractions containing dissociated caseins, and then study the properties of the partly dissociated caseins, as well as of native caseins enriched with β-casein. It was hypothesized that dissociation and enrichment of $CaPO_4$ and/or β-casein with native casein micelles would create protein particles structurally different from those originally found in milk.

In addition to a detailed compositional analysis, the modified casein micelles were investigated by small-angle X-ray scattering (SAXS), as this method has proven to be a good tool to investigate small inner structural changes (De Kruif, 2014; Ingham et al., 2016; Jan Skov Pedersen, Møller, Raak, & Corredig, 2022; Raak, Pedersen, & Corredig, 2023). This work is important for the future development of new functional and nutritional ingredients created from recombination of new dairy streams, created through new technologies.

2. Materials and methods

2.1. Sample preparation

Casein micelle suspensions were prepared using ultracentrifugation as previously described (Møller et al., 2023). Their dissociation was induced by mild acidification and incubation at 4 °C or 22 °C (Møller et al., 2023). In short, 0.2 mg/mL Na Azide was added to pasteurized (75 °C, 15 s) and non-homogenized skim milk to prevent bacterial growth. Casein micelles were separated from whey by ultracentrifugation (100,000×*g*, 1 h, 22 °C, Beckman Coulter); the serum supernatant was discarded and the pellet was resuspended in the same volume of milk serum, prepared by ultrafiltration of skim milk as described elsewhere (Coşkun, Wiking, Yazdi, & Corredig, 2022). This resulted in micellar casein suspensions depleted of serum proteins, but with an expected comparable mineral composition and volume fraction (approximately 0.1) to that of native skim milk. Micellar casein suspensions were then incubated overnight at either 4 °C or 22 °C, at pH 6.8 and pH 6.0, the latter following the addition of 0.35% w/w Glucono-δ-lactone (GDL). The soluble phases from pH 6.8 and 6.0 suspensions were then separated from the micellar phases by ultracentrifugation at either 4 °C or 22 °C and the supernatants collected. The fractions were named S6.8–22 °C (indicating supernatant from micellar suspensions with pH 6.8 and incubated at 22°C), S6.8–4 °C (supernatant, pH 6.8, 4°C), S6.0–22 °C (supernatant, pH 6.0, 22 °C), S6.0–4 °C (supernatant, pH 6.0, 4°C). Control micellar suspensions increasingly acidified from pH 6.8 to pH 5.6 were also prepared for SAXS measurements by addition of varying concentration of GDL, up to 0.40% w/w.

The centrifugal supernatants (S6.8–22 °C, S6.8–4 °C, S6.0–22 °C, S6.0–4 °C) were then used for redispersing pelleted casein micelles separated by centrifugation at native pH and 22 °C (as explained above), to create β-casein- or β-casein + Ca-enriched micellar suspensions. The recombined micellar suspensions were named as follows: 1) *Control* (micelles + S6.8–22 °C), 2) *Control* + *Ca* (micelles + S6.0–22 °C), 3) *β* + (micelles + S6.8–4 °C) and 4) *β*+*Ca* + (micelles + S6.0–4 °C).

2.2. Protein analyses

Protein composition was measured by Reverse Phase High-Performance Liquid chromatography (RP-HPLC) followed by Electrospray Ionization Mass Spectrometry (ESI-MS), on an Agilent LC/MSD XD (G6135B, Agilent Technologies, Santa Clara, CA, USA). Samples (150 μL) were mixed with 450 μL denaturing solution (6 M Guanidinium Hydrochloride, 5.37 mM Sodium Citrate in 100 mM Bis-Tris, pH 6.8) and 12 μL 1 M 1,4-Dithioerytritol (DTE). The samples were then filtered through 0.2 μm filters (Whatman™ Mini-UniPrep® syringeless filters, PTFE membrane) before injection onto a Jupiter® C4 column (250 × 2.00 mm, 5 μm particle size, 300 Å pores; Phenomenex®). The column was kept at 40 °C and buffer A (0.05% v/v TFA in milliQ water) and B (0.05% v/v TFA in Acetonitrile) were used with the following sequence: Solvent B eluted with 33% at 0 min, 44.3% after 40 min and returned to 33% after 41 min. UV absorbance was measured at 214 nm with a diode array detector (DAD, instrument no. G7117C, Agilent). The running flow was 0.3 mL/min and the injection volume was 6 μL. Masshunter 10 Bioconfirm software (Agilent) was used for deconvolution. The deconvoluted masses were then assigned to a milk protein specific in-house database. The detailed data processing method is described elsewhere (Thesbjerg, Johansen, Larsen, & Poulsen, 2022).

The absolute concentration of protein in micellar suspensions reconstituted in permeate was ~28 mg/mL as determined by measuring Nitrogen content with Dumas method (Gerhardt Dumatherm®, Gerhardt GmbH&Co, Konigswinter, DE) and using a conversion factor of 6.38. In the original micellar suspensions, the β-casein constituted ~39% of the total casein content, estimated from the RP-HPLC elution profile. The concentration of β-casein in supernatants from cold-stored (S6.8–4 °C) and acidified micellar suspensions (S6.0–4 °C) was

likewise determined (elution profile by RP-HPLC), and an absolute β-casein concentration was estimated by relating to the protein concentration determined by Dumas.

2.3. Mineral analysis

Ca and P were analyzed and quantified by Inductively Coupled Plasma (ICP) Mass Spectrometry (MS) on an Agilent 7900 instrument. Prior to mineral analyses by ICP-MS, samples were acid hydrolyzed and digested in an Anton Paar Multiwave 3000, using the 64MG5 rotor. A volume of 0.3 mL of sample was mixed with 0.43 mL of concentrated HNO_3 in Wheaton® glass vials (product nr. 41,214) sealed with plastic seals (product nr. 41,186) and screwable caps (product nr. 41,188). The microwave power was increased to 800 W within 10 min, held for 10 min, decreased to 0 W, and held for 10 min. Temperature limit was set to 145 °C. After digestion, approximately 4.5 mL of MilliQ water was added to a final dilution factor of 130–180. The sample weight before digestion and after dilution was noted to calculate the exact dilution factor. Analyses of Ca and P were conducted in He mode as specified elsewhere (Møller et al., 2023). Calibration standard solutions were prepared in the concentration range 0.5–25 mg/L of Ca and P. Yttrium was used as internal standard at 5 mg/L. All standard solutions were purchased from Certipur®.

2.4. Small-angle X-ray scattering

SAXS was performed on a modified version of a NanoSTAR (Bruker AXS) pinhole camera installed on a rotating anode X-ray source (MacScience Cu with a 0.1 × 0.1 mm effective source size operated at 1 kW) with a two-dimensional position-sensitive gas detector (Våntec 500). The instrumental details are described elsewhere (Lyngso & Pedersen, 2021; J. S. Pedersen, 2004). Scattering intensities are given on an absolute scale as a function of the modulus of the scattering vector q ($q = 4\pi\sin(\theta)/\lambda$, where 2θ is the scattering angle and λ is the wavelength of the incoming X-rays). The scattering intensity was recorded at a low-q setup with a sample-to-detector distance of 106 cm giving a q vector range of 0.0045–0.23 $Å^{-1}$. Scattering variations were corrected automatically by scaling to the intensity behind the semitransparent beamstop, and scattering intensities were scaled to the scattering of water to convert data to absolute scale. All scattering data from skim milk, micellar suspensions incubated at 4 °C or 22 °C, and various pH values (pH 6.8–5.6), and recombined micelles were background subtracted using the scattering contributions from their corresponding centrifugal supernatants. The scattering from micellar suspensions that were redispersed at room temperature, and their corresponding centrifugal supernatants, were recorded at 21 °C. The scattering from micellar suspensions that were redispersed at 4 °C and their corresponding centrifugal supernatants were recorded at 4 °C. Recombined micellar suspensions and their corresponding centrifugal supernatants were recorded at 21 °C.

2.5. Statistical analysis

Values from experimental replicates were averaged and statistically analyzed with *Students' t-test*. Statistical difference between averaged values was determined for p-values below 0.05.

3. Results and discussion

3.1. Protein and mineral composition of the supernatants and the enriched micelle suspensions

Casein micelles were separated from whey proteins using centrifugation, and resuspended in whey protein free serum (permeate). The protein concentration was ~28 mg/mL. The resuspended micelles were then incubated at either 22 °C or 4 °C, pH 6.8 or 6.0 to cause different degrees of dissociation and obtain serums with varying protein composition, as previously described in detail (Møller et al., 2023). It was previously shown that this resuspension process caused a limited release of caseins from the casein micelles amounting to approximately 2.6% casein dissociation, corresponding to ~0.7 mg/mL soluble caseins. Moreover, the control supernatant (S6.8–22 °C) contained 317 ± 23 mg/L Ca and 427 ± 53 mg/L P. On the other hand, the supernatant separated from micellar suspensions that were incubated at 22 °C but at lower pH (S6.0–22 °C), were enriched of Ca and P, due to the solubilization of the CCP at this pH, but showed no difference in the degree of casein dissociation compared to those at pH 6.8. Hence, this supernatant was equivalent in protein concentration to that of the control (~0.7 mg/mL), but enriched in Ca and P (582 ± 88 mg/L and 627 ± 121 mg/L, respectively) (Møller et al., 2023). As expected, this was not the case for casein micelle suspensions incubated at 4 °C, which showed an increase in β-casein in the soluble phase, corresponding to ~23% β-casein dissociation from the micelles at pH 6.8. The total casein concentration in this supernatant (S6.8–4 °C) was estimated to about 3 mg/mL, whereof 2.5 mg/mL was β-casein. Similarly to what was observed for the samples incubated at 22 °C, the supernatants at 4 °C and pH 6.0 (S6.0–4 °C) had a similar protein dissociation, but higher Ca and P levels compared to S6.8–4 °C (Møller et al., 2023). It is important to point out that the α_S-casein dissociation remained constant regardless of temperature or pH, as already reported (Møller et al., 2023). We note that the release of protein from the micelles is not large enough to give a significant change of the average electron density of the serum phase and therefore it does not change the contrast of the components within the casein micelles.

In summary, by mild acidification and incubation at different temperatures, it was possible to obtain serum phases either enriched in $CaPO_4$ or enriched in β-casein, or both. These sera were then recombined with pelleted casein micelles, separated from the whey protein by ultracentrifugation. The recombination resulted in micellar suspensions enriched in β-casein and varying in soluble ions. The compositions of the reconstituted suspensions as well as their corresponding soluble phase, after overnight equilibration at 22 °C, are summarized in Table 1. The samples were kept at 22 °C to facilitate β-casein re-association in the casein micelles. It is known that β-casein dissociates at low temperature, but this dissociation is reversible (Creamer et al., 1977). As expected, while the control and the suspensions with added β-casein at pH 6.8 (β+) maintained their native pH, the micelles recombined with acidified supernatants (*Control* + *Ca* and β+*Ca*) showed a decrease in pH to 6.3 and 6.2, respectively. Although the total Ca and P content of the samples did not show a significant difference within the experimental error, the acidified recombined suspensions (*Control* + *Ca* and β+*Ca*) showed a higher amount of soluble Ca and P compared to the non-acidified recombinations (*Control* and β+).

The concentration of soluble Ca and P for the *Control* and β + reconstituted at pH 6.8 ranged from 300 to 370 mg/L Ca and 394–480 mg/L P. For *Control* + *Ca* and β+*Ca*, the soluble Ca and P concentrations ranged between 550 and 600 mg/L Ca, and ~550 mg/L P. Micellar Ca and P was estimated from the difference between the total concentration and that recovered in the centrifugal supernatant. All samples showed similar micellar Ca concentrations ranging between 900 and 1060 mg/L Ca, although micellar Ca tended to be higher for *Control* + *Ca* and β+*Ca* (Møller et al., 2023). The latter was likely because of Ca association to the Ser-linked PO_4-groups of β-casein, which was also reflected by the increased micellar P levels of this sample (~700 mg/L P). The P levels for the remaining suspensions, *Control*, *Control* + *Ca* and β + were ~550 mg/L P (Møller et al., 2023).

The differences in the casein dissociation in the various recombined fractions were measured by RP-HPLC. Fig. 1 shows the elution of the caseins present in the supernatants resulting from the dissociation, before their recombination with native micelles (Fig. 1B–E, full lines), and the supernatants after recombination (Fig. 1B–E dashed lines). Fig. 1A shows the elution profile of the total protein composition of the

Table 1
Values of pH and Ca and P concentrations in the recombined casein micelles suspensions as well as in the corresponding serum phase, separated after ultracentrifugation at 22 °C. The values are averages of at least two independent experiments ± standard deviations.

Name	Treatment	pH	Total (mg/L)		Soluble (mg/L)		% Soluble		Micellar (mg/L)	
			Ca	P	Ca	P	Ca	P	Ca	P
Control	Micelles+ S6.8–22 °C	6.80 ± 0.01	1245 ± 90	949 ± 23	300 ± 26[a]	394 ± 22[a]	25 ± 3	41 ± 3	893 ± 47	567 ± 36
Control + Ca	Micelles + S6.0–22 °C	6.30 ± 0.01	1447 ± 338	1072 ± 240	582 ± 99[a,b]	567 ± 86[a,b]	39 ± 5	50 ± 5	944 ± 338	577 ± 203
β+	Micelles + S6.8–4 °C	6.80 ± 0.01	1218 ± 184	964 ± 139	371 ± 53[a,b]	479 ± 49[a,b]	29 ± 1	47 ± 2	907 ± 162	536 ± 102
β+*Ca*	Micelles + S6.0–4 °C	6.20 ± 0.04	1465 ± 259	1126 ± 192	555 ± 34[b]	535 ± 27[b]	35 ± 4	43 ± 4	1057 ± 112	700 ± 77

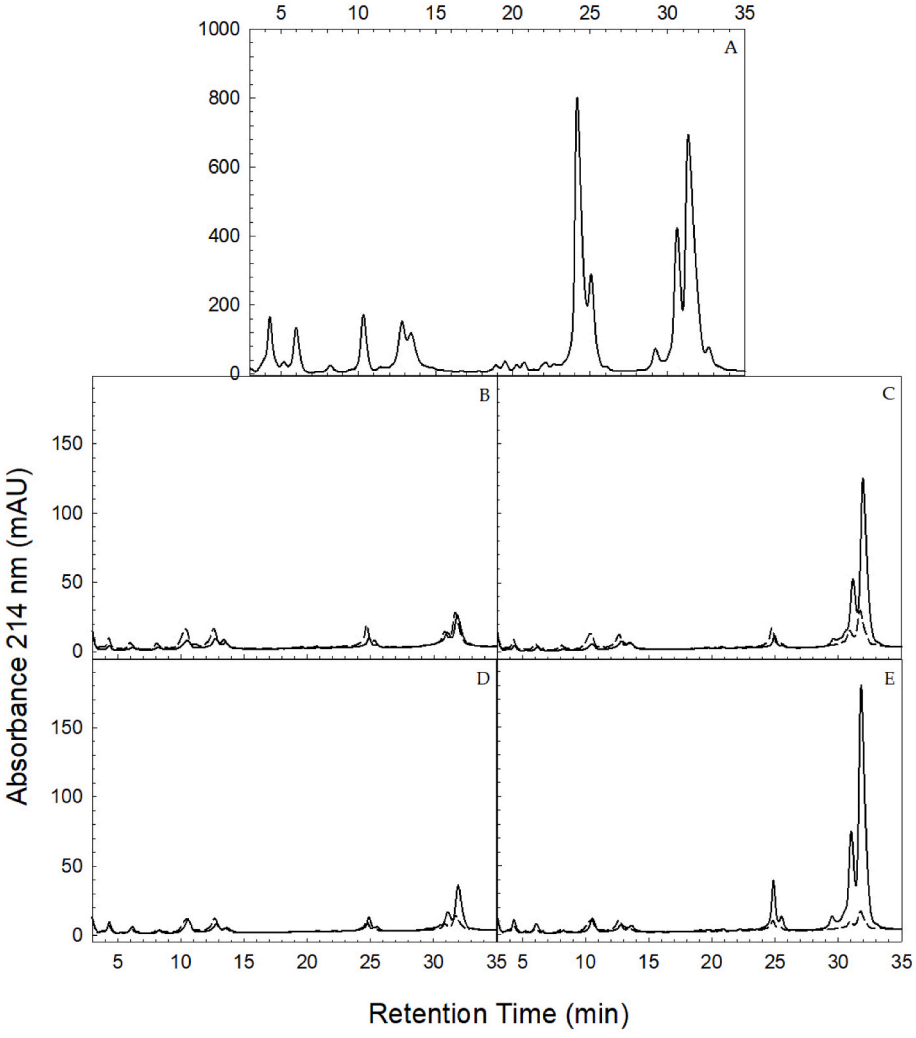

Fig. 1. Representative elution chromatograms from various recombined samples. A: Control recombined micelles (micelles + S6.8-22 °C). B-E supernatants of recombined micelles (dashed lines) and their serum phases before recombination (solid lines), pH 6.8 (B,C) and acidified (D,E). **B**: Control (micelles + S6.8–22 °C); **C**: β+ (micelles + S6.8–4 °C); **D**: Control + Ca (micelles + S6.0–22 °C); **E**: β+Ca (micelles + S6.0–4 °C). Note differences on the y-axes.

Control suspension, which was prepared by recombining native casein micelles with S6.8–22 °C. All recombined suspensions had comparable protein compositions and contained all the main casein proteins (Fig. 1A). It was possible to resolve most genetic variants and phosphorylation degrees for all four caseins. The first three peaks eluting before 11.5 min were assigned to genetic variants A, B, and E of κ-casein containing varying degrees of glycosylation (glyc) and phosphoserines (P); the fourth (split-)peak eluting after 12–14 min was assigned to α_{S2}-casein, phosphorylated to various degrees which created the split peak; α_{S1}-casein 8 - 9 P eluted between 23 and 26 min. Various genetic isoforms of β-casein eluted between 29 and 33 min, whereof isoform B eluted in the small peak at 29 min, variant A1 eluted at 30.5 min and variant A2 at 32 min coeluting with variant I. The major whey proteins, β-LG and α-LA, which elute later in the chromatogram, were not detected in these samples (Møller et al., 2023) confirming the successful removal of these components.

The comparison between the sera before and after recombination (Fig. 1B–E) clearly demonstrated that in all samples there was a significant re-association of the proteins. Indeed, the additional protein added to the native micelles after re-association was no longer in the

supernatant, but was recovered in the sedimentable fraction, regardless of the treatment applied. The added β-casein was then either recombined or co-sedimented with the micelles.

3.2. SAXS of depleted and enriched casein micelles

3.2.1. SAXS of β-casein and $CaPO_4$ depleted micelles

To observe changes in the structure of depleted and recombined micelles, SAXS analysis was performed. Indeed, SAXS is a suitable means for probing changes at length scales corresponding to those of the protein-protein and protein-CCP nanocluster interactions present in the inner structure of casein micelles (Jan Skov Pedersen et al., 2022). The range of q used in these experiments probed two of the main scattering features of the casein micelles, which can be seen in Fig. 2. At q values from 0.01 to 0.02 $Å^{-1}$, the scattering feature is attributed to the presence of intermediate structures within the micelles, composed of casein proteins and CCP nanoclusters (See example Bouchoux, Gesan-Guiziou, Perez, & Cabane, 2010; Ingham et al., 2016; Jan Skov Pedersen et al., 2022). These intermediate structures scatter because they are separated in space by serum-filled cavities. It has previously been hypothesized that these structures may be stabilized by β-casein (Dalgleish, 2011; Dalgleish & Corredig, 2012). At a smaller length scale (higher q values), the protein and CCP nanoclusters, collectively referred to as subparticles, dominate the scattering signal. The protein structures were modelled as star-like protein particles and the CCP nanoclusters as oblate ellipsoidal particles (Jan Skov Pedersen et al., 2022). The combined scattering contribution from the subparticles and interactions between them give rise to the shoulder at $q = 0.08$ $Å^{-1}$ (Jan Skov Pedersen et al., 2022). The plateau forming at $q = 0.007$ $Å^{-1}$ was caused by the beamstop shadowing and pinhole smearing of the signal, as described in Pedersen et al. (2022).

Fig. 2A shows the scattering intensity as a function of q for skim milk and the control micellar suspensions incubated at 22 °C or 4 °C, at native pH (pH 6.8). The scattering from casein micelles incubated at 22 °C (empty triangles, Fig. 2A) was identical to the scattering from the original skim milk (filled circles, Fig. 2A). This demonstrated that the separation of the micelles and their resuspension with their original serum did not affect their structure at this length scale. However, this was not the case for the SAXS pattern of the micelles incubated at 4 °C. These samples (inverted triangles, Fig. 2A) differed from native micelles (pH 6.8, 22 °C) in two ways: 1) They showed a decreased intensity in the intermediate q region ($q = 0.01$–0.02 $Å^{-1}$) (Fig. 2B) and 2) the scattering

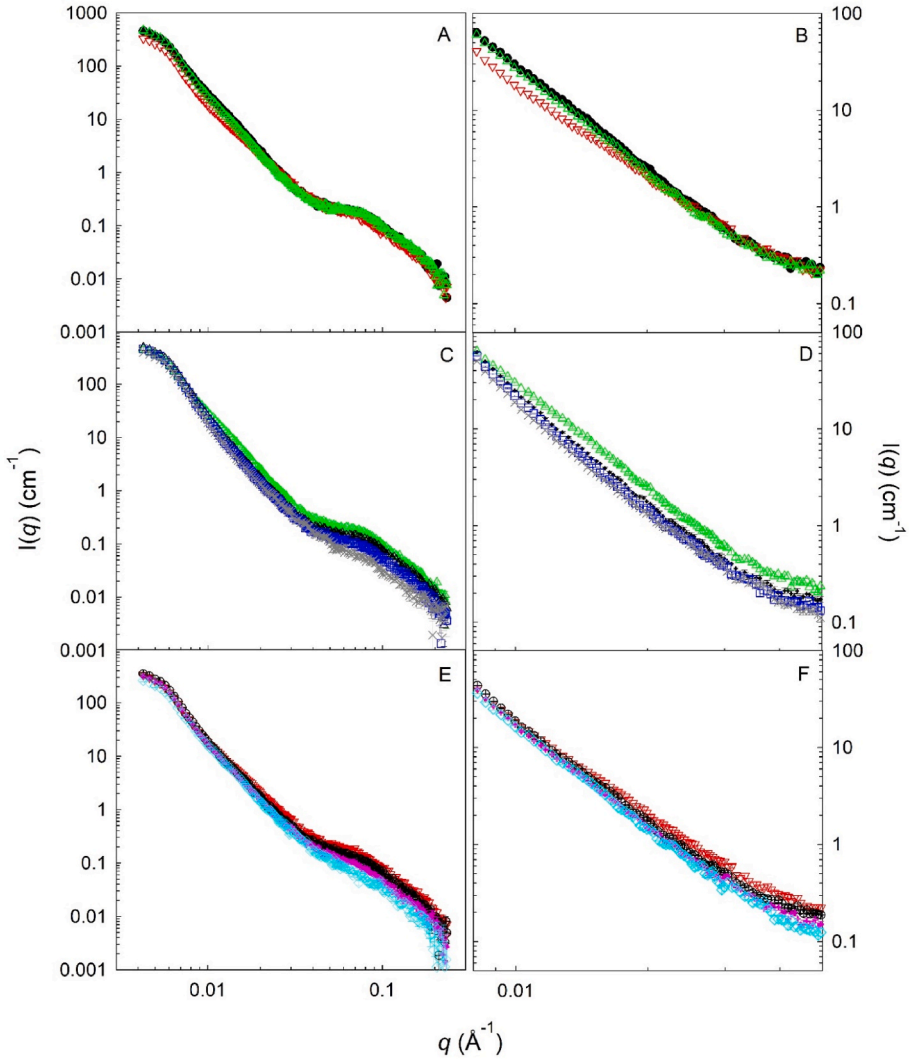

Fig. 2. SAXS of skim milk micelles, control and acidified micellar suspensions, subtracted by their corresponding centrifugal supernatants. B, **D** and **F** are enlargements of **A**, **C** and **E**, respectively, in the q-range 0.008–0.05 $Å^{-1}$. **A,B**: Comparison of skim milk (circles, filled) and micellar suspensions incubated at 22 °C (triangles, green) or 4 °C (inverted triangles, red) at pH 6.8. **C,D**: Micellar suspensions incubated at 22 °C, pH 6.8 (triangles, green), pH 6.3 (cross, black), pH 5.9 (squares, blue) and pH 5.6 (X, grey); **E,F**: Micellar suspensions incubated at 4 °C, pH 6.8 (upside triangles, red), pH 6.4 (crossed circles, black), pH 6.1 (+ , pink) and pH 5.9 (diamonds, cyan). Error bars were omitted to better visualize data points.

intensity beyond $q = 0.08$ Å^{-1} decreased slightly. The decreased intensity in the intermediate q-region meant that the inner structure of the micelle, at the length scale of the intermediate particles, became more homogeneous compared to skim milk and control micelles. Since the pH and CCP concentrations were the same within statistical error between the micellar suspensions incubated at 22 °C or 4 °C (Table 1), the decreased scattering contrast must be attributed to the loss of β-casein. This reinforced the hypothesis that a portion of the micellar β-casein may be responsible for the stabilization of internal scattering structures, surrounded by less dense, serum filled areas. The difference in the scattering in this q range indicated that with less β-casein, the boundaries between casein/mineral-dense areas and serum-filled cavities are no longer as defined. The slight decrease in intensity at higher q-values for $q > 0.08$ Å^{-1} for the 4 °C sample (Fig. 2A) indicated that there is a small rearrangement of the protein and CCP nanoclusters. Interestingly, previous work (Marchin et al., 2007) found no difference in the SAXS from a control micellar suspension (prepared in the same way as the resuspended casein micelle suspension at 22 °C in this work) and a β-casein depleted micellar suspension, however, this discrepancy could be explained by the fact that Marchin and coworkers measured the X-ray scattering from both micellar suspensions at room temperature, whereas we measured the cooled micellar suspension at 4 °C. The work by Takagi, Nakano, Aoki, & Tanimoto, 2022, in agreement with this work, showed a slight intensity decrease in the intermediate q-region upon cooling from 40 °C to 10 °C; however, their scattering curve also showed a slight increase in the q region 0.2–0.7 Å^{-1} (Takagi et al., 2022), which we did not observe. The discrepancy at high q is likely related to differences in background subtraction. Indeed, the authors of Takagi et al. did not specify the background used for subtraction.

Control experiments were performed to understand the effect of acidification, by creating a series of micellar suspensions incubated at the two temperatures (22 °C and 4 °C) and acidified to various pH values, in the pH range 6.8 - 5.6. This allowed the evaluation of the scattering changes for control micellar suspensions as a result of acidification. Fig. 2C–D and 2E-F show the SAXS data of micellar suspensions from pH 6.8–5.6 incubated at 22 °C or 4 °C, respectively. There was a gradual loss of intensity at the high-q shoulder, at both incubation temperatures (Fig. 2C–F). This decrease was attributed to the release of CCP from the micellar phase.

There was also a clear difference in the scattering pattern in the intermediate q region ($q = 0.01$–0.02 Å^{-1}) between the micellar suspensions acidified at the two temperatures (Fig. 2D vs 2F). While the casein suspensions incubated at 22 °C (Fig. 2D) gradually lost the scattering intensity at intermediate q as a function of lower pH, there was a smaller change in this intermediate bump for the suspensions incubated at 4 °C (Fig. 2F). Previous work did not show significant caseins' dissociation in micellar suspensions acidified to pH 6.0 at 22 °C (Møller et al., 2023), therefore, the sudden loss of intensity at the intermediate q region going from pH 6.8 (triangles, Fig. 2D) to 6.3 (crosses, Fig. 2D), and further down to pH 5.6 (X, Fig. 2D) in these suspensions, must be explained not by a loss of protein, but by a change in the overall protein charges, due to an increase in H^+ concentration. The increase in H^+ concentration was expected to decrease electrostatic repulsion within the casein micelle, thus allowing for a more homogeneous structure at the intermediate length scale, similar to the effect of β-casein dissociation as observed from Fig. 2B, explaining why we do not see such a drastic loss of intensity going from pH 6.8 (inverted triangles, Fig. 2F) to 6.4 (crossed circles, Fig. 2F).

These findings corresponded well with the findings of Ingham et al. (2016) who also observed a gradual loss of the intensity in the intermediate- and high-q region upon acidification at room temperature. More recent work (Fan, Fehér, Hettinga, Voets, & Bijl, 2024) studied the effect of pH on recombined casein micelles by SAXS and fitted the data with the newly developed model by Pedersen et al. (2022). The parameter termed $n_{intermediate}$ is a measure of the scattering contribution from intermediate particles relative to the overall scattering, and it manifests itself in the intermediate q region (0.008–0.02 Å^{-1}). The cited work found a decrease in the value of $n_{intermediate}$, corresponding to an intensity decrease of the intermediate q region, relative to the overall scattering, thus agreeing with the present findings (Fig. 2D).

Fig. 3 shows the SAXS profiles of the four casein micelles treatments, after the resuspension with serum with and without β-casein enrichment, and at the two pH values. In addition, a Kratky plot in a log-log representation was also included in Fig. 3B. A Kratky plot is a different representation of scattering data where the scattering intensity is multiplied q^2 and plotted as a function of the q vector modulus. This was included to visually emphasize the differences in scattering at the intermediate- and high-q regions. The scattering from β-casein enriched micelles at pH 6.8 (β+, filled inverted triangles, Fig. 3) was similar to its corresponding control (*Control*, filled triangles, Fig. 3). The slight difference in the scattering intensity between the two suspensions can be attributed to slight differences in concentration as a result of the resuspension step. The striking similarity between the two SAXS patterns demonstrates that the enrichment by β-casein did not affect the inner micellar structure probed within this q region. The micelles reconstituted with the serum phases acidified to pH 6.0, and therefore enriched in Ca and P, namely *Control + Ca* (filled squares, Fig. 3) and β+*Ca* (filled diamonds, Fig. 3), maintained a decreased pH of 6.3 and 6.2, respectively. They also showed a similar SAXS intensity pattern among each other, but different from the micelles at pH 6.8 (*Control* and β+), with an overall lower intensity at both the intermediate and high-q regions, and a steeper slope at the intermediate region. The difference is quite evident in the Kratky plot (Fig. 3B). These results were consistent with what was previously shown in Fig. 2, namely that the decreased intensity in the intermediate q-region was related to a decreased pH.

By looking at both resuspended micelles, at pH 6.8 and 6.3/6.2, it was concluded that β-casein enrichment did not affect the scattering pattern of the suspensions, in either of the pH conditions. The decrease of scattering at this intermediate q-range was explained by the decreased overall charges at the lower pH value, which may cause a reduction of the protein-protein and protein-CCP charge repulsion at the interface between serum filled cavities and the material dense areas (= protein and mineral dense areas). In other words, the pH decrease may cause an overall 'relaxation' of the interface between the intermediate structures and the void volumes.

The protein analysis (Fig. 1) of the serum after reconstitution showed that the majority of the β-casein added in the suspensions was no longer recovered in the soluble phase after recombination, but instead recovered in the colloidal phase. However, the location of the enriched β-casein is not known. The concentrations of added β-casein (present in S6.8–4 °C and S6.0–4 °C) was around 3–5 mg/mL, hence, above its critical micellar concentration (Moitzi, Portnaya, Glatter, Ramon, & Danino, 2008; O'Connell, Grinberg, & De Kruif, 2003). Fig. 4 shows the SAXS signal for the supernatants separated at pH 6.8 (S6.8–4 °C), measured at temperatures ranging from 4 to 35 °C. The background contribution from the scattering of permeate (protein free serum) was subtracted in all cases. The background did not vary in response to temperature increase (data not shown). When measured at 4 °C, the data points' statistics were poorer (larger error bars) because the scattering from S6.8–4 °C, measured at 4 °C, was nearly equal to the scattering from UF permeate, the background. This showed that the β-casein is monomeric at this temperature and therefore did not scatter at appreciable levels. Upon temperature increase, a Guinier region formed at q ~0.02 Å^{-1} (Fig. 4) indicative of β-casein self-assembly. A Guinier region is a region at low q, where the data follows a linear behavior in a plot of $\ln(I(q))$ vs q^2. The slope provides information of the particle size in terms of a radius of gyration. The investigation of these structure was not within the scope of the present work, nonetheless, the formation of a Guinier-region is indicative of formation of well-defined particles. It is then likely that the soluble β-casein in β-casein enriched supernatants (S6.8–4 °C and S6.0–4 °C) was prone to associating and self-assembling during the mixing at 22 °C with the casein micelles. Indeed, Fig. 1

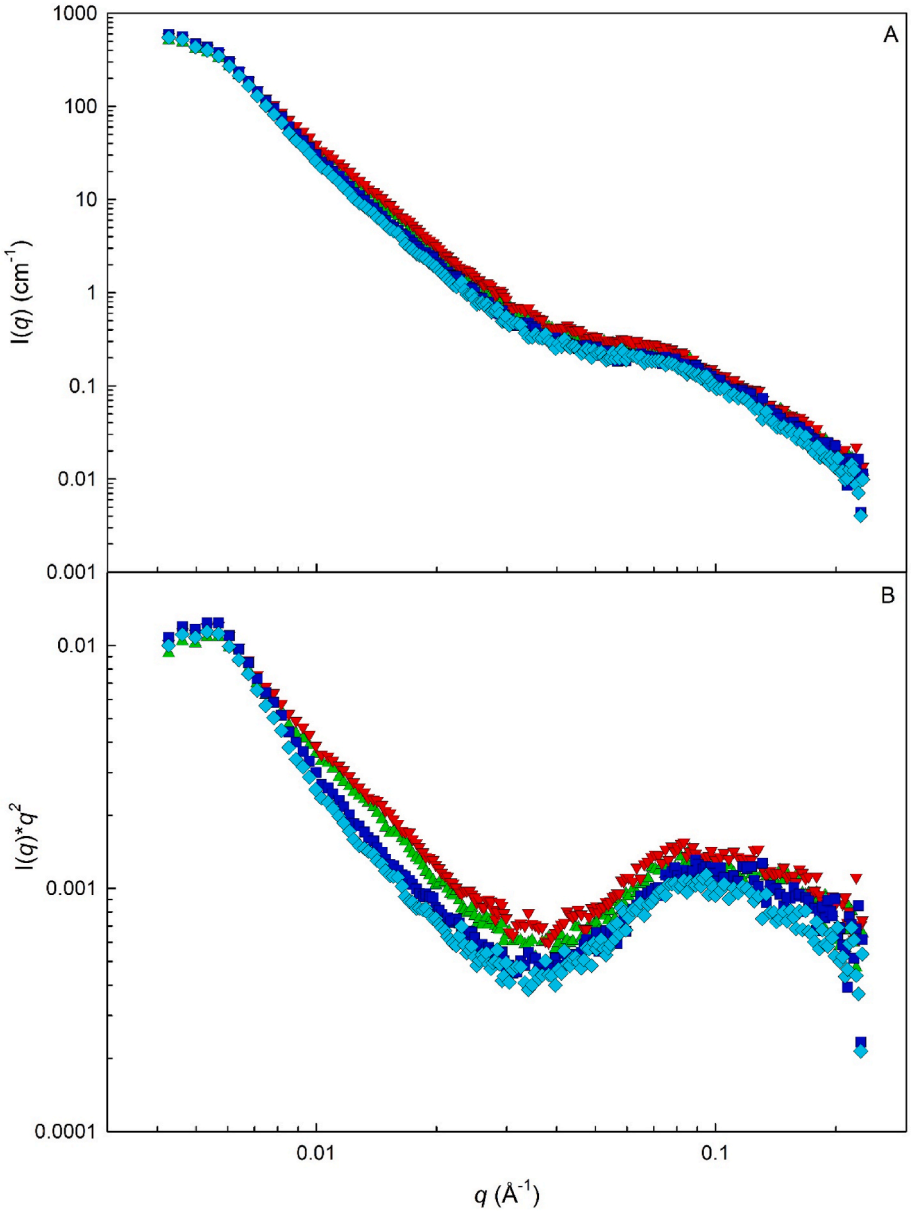

Fig. 3. SAXS data from recombined casein micelles with enriched supernatants S6.8–22 °C (Control, triangles, green), S6.8–4 °C (β+, downward triangles, red), S6.0–22 °C (Control + Ca, squares, blue), S6.0–4 °C (β+Ca, diamonds, cyan). A) log-log plot; B) Kratky plot in log-log representation. Error bars were omitted to better visualize differences in scattering between samples.

showed that the supernatants after the enrichment only contained trace amounts of β-casein, hence most of it was in the colloidal phase. However, the presence of self-assembled β-casein micelles cannot be fully ruled out as the scattering signal from these would contribute at the intermediate q-range, and with very low intensity.

The scattering from the centrifugal supernatants after enrichment (measured at 22 °C) were compared with the supernatants before recombination, which were measured at 4 °C (Fig. 5). Only the soluble phase of the *β+Ca* suspension (filled diamonds, Fig. 5) contained significantly more Ca and P than the others (Table 1), however, this did not affect the scattering data. There was no structure present in the supernatants before recombination (S6.8–4 °C, S6.8–22 °C, S6.0–4 °C and S6.0–22 °C, empty symbols in Fig. 5). In the case for the supernatants isolated at 4 °C (S6.8–4 °C and S6.0–4 °C, inverted triangles and diamonds respectively, Fig. 5) the lack of structure was due to the temperature at which the scattering was measured, 4 °C, thus the caseins were present in their dissociated form, as seen from Fig. 4. In the case for the supernatants isolated at 22 °C, whose X-ray scatterings were recorded at 22 °C, there were only trace amounts of caseins present, as evident from Fig. 1B–E and this was therefore the reason for the lack of noteworthy scattering features (Fig. 5). The picture was different when looking at the scattering data from supernatants isolated after recombination; in agreement with the similarities in their composition, the four scattering data sets of supernatants after recombination were similar in shape. The main difference in scattering among the supernatants isolated after recombination was found between the acidified and non-acidified sera: Scattering from the acidified sera (filled squares and diamonds, Fig. 5) were lower in intensity compared to non-acidified sera (filled triangles and inverted triangles, Fig. 5), as was for the enriched micelles (Fig. 3). Interestingly, after recombination, although much less protein was present in the supernatants, as shown in Fig. 1, the scattering data form a plateau at lower q (=0.007 $Å^{-1}$) suggested the presence of finite-sized unsedimented protein aggregates.

The aim of the present work was to evaluate if changes in the internal

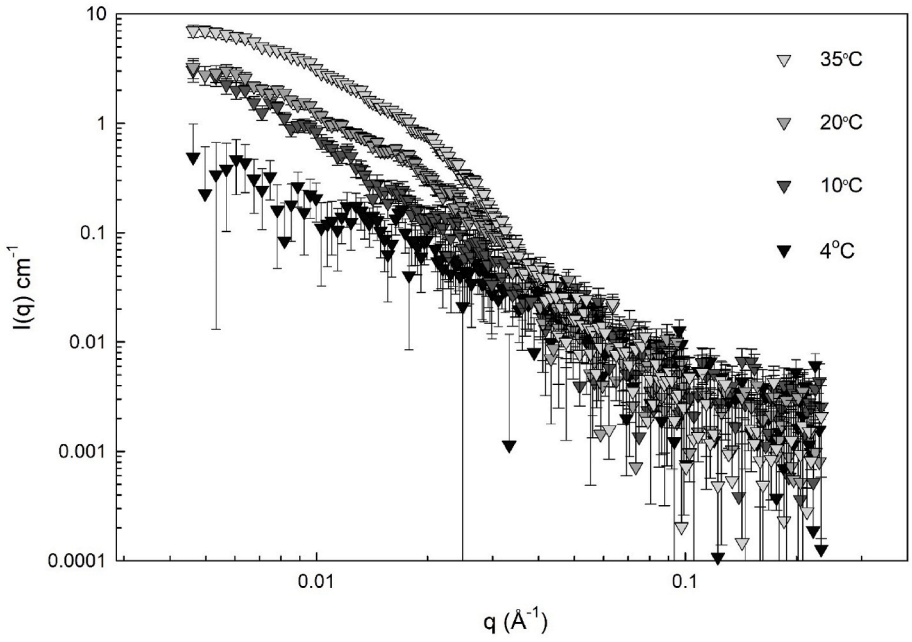

Fig. 4. Scattering from β-casein micelles, 4–35 °C. SAXS of the supernatant isolated from micellar suspension at 4 °C, pH 6.8, measured at 4 °C, 10 °C, 20 °C and 35 °C.

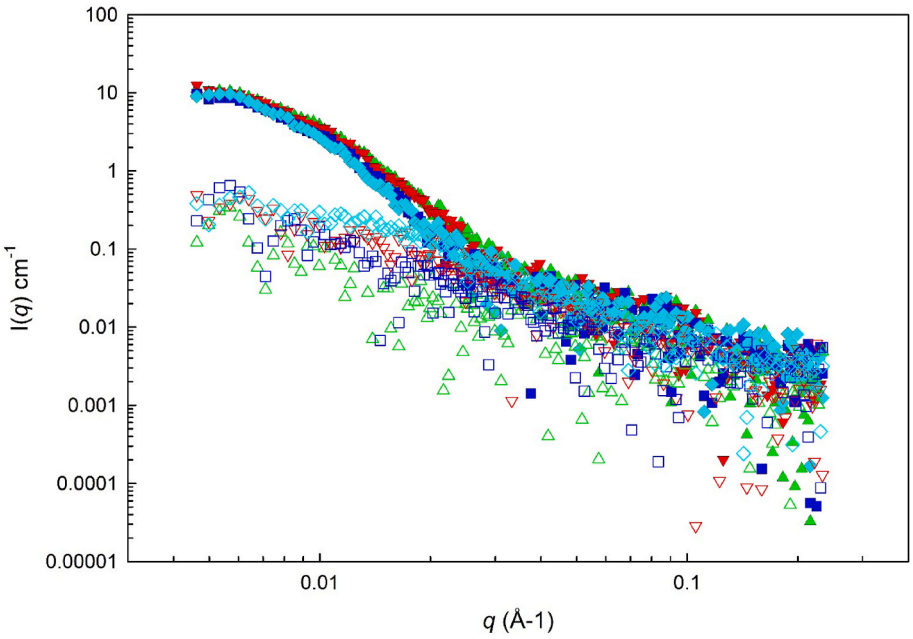

Fig. 5. SAXS of supernatants isolated before and after recombination with enriched supernatants. Empty symbols: Supernatants isolated before recombination, S6.8–22 °C (triangles, green), S6.8–4 °C (inverted triangles, red), S6.0–22 °C (squares, blue) and S6.0–4 °C (diamonds, cyan). **Filled symbols**: Supernatants isolated after native micelles' recombination with supernatants S6.8–22 °C (triangles, green), S6.8–4 °C (inverted triangles, red), S6.0–22 °C (squares, blue) and S6.0–4 °C (diamonds, cyan). Error bars were omitted to better visualize data points.

structure of the casein micelles occurred after slight compositional modifications using pH and cooling. In addition, native casein micelles were enriched with β-casein and ions and the structure of these micelles was also probed by SAXS.

Comparison of micelles acidified from pH 6.8 down to pH 5.6 at 22 °C and 4 °C showed that a loss of β-casein, as a consequence of cooling, and without acidification, resulted in a more homogenous inner structure at the intermediate length scale (q = 0.01–0.02 Å^{-1}). Gradual acidification resulted in a gradual decrease of the high-q shoulder intensity (q = 0.08 Å^{-1}) which was a direct cause of loss of CCP subparticles. Moreover, the increase in H^+ concentration reduced the heterogeneity at the intermediate length scale by causing a decrease in the charge repulsion between intermediate structures and proteins (Fig. 2).

When native micelles were enriched with β-casein, the additional β-casein associated to the colloidal phase (Fig. 1), however, no change was observed in the X-ray scattering data of control micelles and micelles recombined with β-casein and/or Ca enriched sera (Fig. 3). This would suggest that in the presence of casein micelles, the β-casein interacts with the other protein structures already forming scattering

heterogeneities (so-called 'intermediate structures'), and not further add in an observable manner to the signal intensity. However, the potential of β-casein self-micellization cannot be ruled out since it was shown that the β-casein in the enriched serum, S6.8–4 °C, give rise to scattering with a Guinier region at $q = 0.02$ Å^{-1} with increasing temperatures (Fig. 4). Functionality tests are then needed to further evaluate the spatial distribution of the recombined β-casein.

4. Conclusion

It can be concluded that albeit a dissociation of the β-casein causes interior rearrangements of the casein micelles, in turn, an addition of β-casein to native micelles does not further affect the structure. A decrease in pH also clearly caused a loss of intensity at intermediate *q* affecting the structural heterogeneities' scattering in this *q* range, and caused a change in the slope of the scattering intensity, suggesting that the internal structure becomes more homogeneous, due to a decrease in charge repulsion between the protein moieties. Previous researchers have suggested the existence of water-channels in the casein micelle structure (Bouchoux et al., 2010; Dalgleish, 2011). It has been hypothesized (Bouchoux and Dalgleish) that the protein heterogeneities contain regions of less dense protein stabilizing these water channels. These less dense areas could be constituted by β-casein. The release of β-casein in the serum phase with cooling would then cause a decrease in the heterogeneities, while an enrichment of β-casein would not further contribute to the internal structure. Furthermore, a slight decrease in pH would cause a decrease of electrostatic repulsion, and yet again, would contribute to the smoothing of the structural heterogeneities.

CRediT authorship contribution statement

Thea Lykkegaard Møller: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Søren Bang Nielsen:** Writing – review & editing. **Jan Skov Pedersen:** Data curation, Software, Supervision, Writing – review & editing. **Milena Corredig:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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