Food Research International 173 (2023) 113315

Contents lists available at ScienceDirect

Food Research International

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

Engineering artificial casein micelles for future food: Is casein phosphorylation necessary?

Laurens J. Antuma [a], Isabell Steiner [a], Vasil M. Garamus [b], Remko M. Boom [a], Julia K. Keppler [a,*]

[a] Laboratory of Food Process Engineering, Wageningen University & Research, Bornse Weilanden 9, 6708 WG, Wageningen, Netherlands
[b] Helmholtz Zentrum Hereon, Max-Planck Str. 1, D-21502 Geesthacht, Germany

ARTICLE INFO

Keywords:
Post-translational modification
Dephosphorylation
Hybrid micelles
Calcium phosphate nanocluster
Rennet coagulation
Cheese

ABSTRACT

Industrial-scale production of recombinant proteins for food products may become economically feasible but correct post-translational modification of proteins by microbial expression systems remains a challenge. For efficient production of hybrid products from bovine casein and recombinant casein, it is therefore of interest to evaluate the necessity of casein post-translational phosphorylation for the preparation of hybrid casein micelles and study their rennet-induced coagulation. Our results show that dephosphorylated casein was hardly incorporated into artificial casein micelles but was capable of stabilising calcium phosphate nanoclusters with an increased size through adsorption on their surface. Thereby, dephosphorylated casein formed larger colloidal particles with a decreased hydration. Furthermore, the presence of increasing amounts of dephosphorylated casein resulted in increasingly poor rennet coagulation behaviour, where dephosphorylated casein disrupted the formation of a coherent gel network by native casein. These results emphasise that post-translational phosphorylation of casein is crucial for their assembly into micelles and thereby for the production of dairy products for which the casein micelle structure is a prerequisite, such as many cheese varieties and yoghurt. Therefore, phosphorylation of future recombinant casein is essential to allow its use in the production of animal-free dairy products.

1. Introduction

Cheese, and animal-based food products in general, are associated with sustainability and animal welfare issues, leading to an increase in the demand for nutritious and functional animal-free cheese alternatives. Therefore, recombinant synthesis of milk proteins receives increasing interest (Hettinga & Bijl, 2022; Keppler et al., 2021). This encompasses the expression of genetically modified protein genes in hosts such as bacteria, yeasts, or filamentous fungi. Casein has already been expressed successfully in various host systems and their industrial-scale production for application in food products (e.g. animal-free cheese) may become commercially feasible due to improving technology and decreasing costs (Hettinga & Bijl, 2022; Tubb & Seba, 2021). However, correct post-translational modification of recombinant casein is difficult to achieve, as prokaryotic expression systems have limited ability to provide post-translational modification (Jia & Jeon, 2016) and eukaryotic systems tend to provide additional post-translational modifications that do not occur on the natural protein (Kalidas et al., 2001; Saito et al., 2002).

Phosphorylation is a type of post-translational modification that regulates the techno-functional properties of proteins (Cohen, 2002). Caseins are an example of phosphorylated proteins and constitute about 80% of the total protein in bovine milk (Fox & Brodkorb, 2008). The reference proteins of the α_{s1}-, α_{s2}-, β-, and κ-casein families possess 8, 11, 5, and 1 phosphorylated amino acid(s), respectively (Farrell et al., 2004). Three or more phosphorylated residues together with two or more acidic residues concentrated in a relatively short sequence constitute a so-called phosphate centre of which the caseins possess 2, 3, 1, and 0, respectively (De Kruif & Holt, 2003). The phosphate centres render the α_s- and β-caseins sensitive to precipitation at low calcium concentrations and enable them to interact with calcium phosphate (CaP) nanoclusters, which is crucial to prevent the precipitation of these ions that are present in milk in concentrations in excess of their solubility (Holt, 1997). In contrast, κ-casein is insensitive to calcium and protects the other caseins from precipitation through the formation of a micellar structure (Horne, 1998). Thus, the interaction between the

* Corresponding author at: PO Box 17, 6700 AA Wageningen, Netherlands.
E-mail addresses: laurens.antuma@wur.nl (L.J. Antuma), isabell_steiner@outlook.com (I. Steiner), vasyl.haramus@hereon.de (V.M. Garamus), remko.boom@wur.nl (R.M. Boom), julia.keppler@wur.nl (J.K. Keppler).

https://doi.org/10.1016/j.foodres.2023.113315
Received 18 May 2023; Received in revised form 21 July 2023; Accepted 22 July 2023
Available online 24 July 2023

phosphate centres of caseins and CaP nanoclusters drives the formation of casein micelles and is fundamental to the structural integrity of those micelles.

These unique micellar structures appear in milk as spherical particles of a few hundred nanometres in diameter and have vital biological functions, including transporting considerable amounts of calcium and phosphate to the neonate, regulating nutrient flow into the neonatal intestine by coagulating in the stomach, and preventing pathological calcification of the mammary gland (Fox & Brodkorb, 2008; Holt et al., 2013). For human consumption, casein micelles are essential for the production and texture of cheese. During the production of cheese, the conditions in the calf stomach are mimicked (i.e. decreased pH and rennet addition) to coagulate the casein micelles. The proteolysis of κ-casein by enzymes in rennet results in a loss in steric stabilisation and a reduction in the electrostatic repulsion between micelles (Dalgleish, 1993). Consequently, the destabilised micelles start to aggregate and form a curd that can be further processed into many cheese varieties.

The ionic species in milk are distributed between the casein micelle and the serum phase. Due to their insolubility, about two thirds of the calcium and half of the inorganic phosphate in milk at its native pH are stabilised by casein micelles and thus present in the micellar phase (Gaucheron, 2005). Next to that, about a third of the magnesium and a tenth of the citrate associate with the CaP nanoclusters. The rest is present in the serum phase, along with the fully diffusible concentrations of sodium, potassium, and chloride (Bijl et al., 2013). The distribution of the ions over the phases is dependent on factors such as pH and temperature. The micellar ionic species are of vital importance for casein micelle stability (De Kruif & Holt, 2003) and affect the rennet coagulation properties of milk, where increased levels of micellar calcium phosphate increase the curd firmness (Lucey et al., 2003).

Studies with enzymatically dephosphorylated bovine casein showed that a lack of phosphorylation decreases the calcium-binding capacity of caseins (Yamauchi et al., 1967), which decreases their ability to assemble into casein micelles (Bingham et al., 1972; Pepper & Thompson, 1963; Schmidt & Poll, 1989). However, there is no general agreement on the properties of these micelles; Bingham et al. (1972) observed that casein micelles prepared from dephosphorylated casein were larger than native casein micelles, whereas Schmidt & Poll (1989) reported a decrease in particle size upon an increasing degree of casein dephosphorylation. The latter authors also noted that the micellar casein in these systems interact with increasingly larger amounts of minerals, but did not further study the internal structure of these micelles. Both studies suggest that calcium-binding sites other than phosphate centres are involved in the stabilisation of CaP nanoclusters and the formation of casein micelles from dephosphorylated casein.

While these previous experiments showed that partial dephosphorylation of casein impairs its micelle-forming ability, there is still an ongoing question if unphosphorylated caseins can contribute towards the formation of hybrid micelles (i.e. being partially incorporated into micelles) of unphosphorylated casein and native casein and in this way contribute to curd formation during rennet-induced coagulation. These hybrid micelles could be used to produce hybrid food products, which can encourage a more nuanced transition to animal-free foods and generally have a high consumer acceptability (Banovic et al., 2022). Furthermore, if hybrid micelles can be prepared with dephosphorylated casein, this sets the stage to incorporate other unphosphorylated proteins (e.g. from plants) into hybrid casein micelles, provided that the structure and charge do not hinder the incorporation.

In the present study, we therefore revisit casein micelles from dephosphorylated casein. We aimed to study the formation and properties of artificial casein micelles from mixtures of native casein and dephosphorylated casein, as well as their coagulation behaviour, to assess the suitability of future unphosphorylated recombinant caseins for the production of animal-free cheese. Bovine casein was enzymatically dephosphorylated and hybrid casein micelles were then prepared artificially from native bovine casein and the dephosphorylated casein in various ratios. The properties of these micelles and their rennet coagulation at various pH values are assessed.

2. Materials and methods

2.1. Materials

Bovine sodium caseinate (Lactonat EN, 90.2% protein, of which 41% α_s-caseins, 42% ß-casein, and 17% κ-casein) was kindly donated by Lactoprot (Lactoprot Deutschland GmbH, Kaltenkirchen, Germany). Tris (hydroxymethyl)aminomethane (Tris; 252859), alkaline phosphatase from bovine intestinal mucosa (P7640; ≥10 DEA units mg^{-1}), calcium chloride (C1016), magnesium chloride (M8266), potassium phosphate monobasic (P5379), sodium phosphate dibasic (S7907), citric acid (C0759), potassium hydroxide (1.05033), sodium hydroxide (221465), potassium chloride (1.04936), potassium carbonate (1.04928), potassium sulfate (1.05153), trisodium citrate dihydrate (S4641), magnesium citrate tribasic nonahydrate (63067), hydrochloric acid (1.13386), nitric acid (1.00456), hydrogen peroxide (1.07209), sodium chloride (31434), sodium phosphate dibasic dihydrate (1.06580), citric acid monohydrate (1.00244), ethanol absolute (1.00983), and lactic acid solution (252476) were all bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Osmium tetroxide (19134), 50% glutaraldehyde solution (16316-10), and carbon adhesive tabs (77825-12) were bought from EMS (Electron Microscopy Sciences, Hatfield, PA, USA). Hydrochloric acid solution (7647-01-0) was bought from Actu-All (Actu-All Chemicals B.V., Oss, Netherlands). Tripotassium citrate monohydrate (6100-05-6) was bought from VWR (VWR International bvba, Leuven, Belgium). Recombinantly produced chymosin (CHY-MAX Plus, batch no. 3634543) was obtained from Chr. Hansen Holding A/S (Hørsholm, Denmark). Ultrapure water (MilliQ system, Merck KGaA, Darmstadt, Germany) was used for all experiments.

2.2. Methods

2.2.1. Preparation of dephosphorylated casein

Sodium caseinate (Na-CN) was dissolved in 20 mM Tris in water to a protein concentration of 15 g/L by stirring at 60 °C for 30 min. The pH was adjusted to 8.0 with 1 M NaOH and 0.1 g/L alkaline phosphatase was added, after which the solution was incubated for 7 h at 37 °C. Proteolysis as a result of this incubation could be excluded based on a determination of the proteolytic activity of the enzyme and by monitoring the amount of amino groups during incubation with the o-phthalaldehyde assay (data not shown). The enzyme was then inactivated by heating the solution for 10 min at 80 °C. The liberated phosphate was removed through diafiltration of 1L batches for approximately 6 h at 4 °C by using a cross flow membrane (Vivaflow® 200 PES, 10 kDa MWCO, Sartorius AG, Göttingen, Germany) and a peristaltic pump (Watson Marlow 505S, Watson Marlow Fluid Technology Solutions, Falmouth, UK) set at 300 rpm. The retentate was subsequently lyophilised in a Christ Epsilon 2-10D freeze drier (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). The protein content of the lyophilised material was determined in triplicate with the Dumas nitrogen combustion method (Rapid N exceed®, Elementar Analysensysteme GmbH, Langenselbold, Germany) with a protein conversion factor of 6.38.

2.2.2. Preparation of artificial casein micelles

Artificial casein micelles (ACM) were prepared in singlicate according to Schmidt et al. (1977) with minor adjustments. Native caseinate and dephosphorylated caseinate were separately dissolved in water to protein concentrations of 64.1 g/L by stirring at 60 °C for 30 min. The two solutions were carefully mixed in the following ratios: 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10. Three salt solutions were prepared: solution I contained 445 mM $CaCl_2$ and 75 mM $MgCl_2$ adjusted to pH 6.70 with 0.1 M HCl, solution II contained 165 mM

KH_2PO_4 and 165 mM Na_2HPO_4, and solution III contained 135 mM citrate adjusted to pH 6.70 with 1 M KOH. The casein solution (60 mL) and the salt solutions (10 mL each) were carefully pumped into a jacketed glass vessel at 37 °C containing a starting volume of 56.5 mL water in 60 min to reach final concentrations of 30 mM calcium, 22 mM phosphate, 9 mM citrate, 5 mM magnesium, and 25.6 g/L casein. The solution was continuously and vigorously stirred using a magnetic stirrer. Controlled addition of the solutions was achieved by using syringe pumps (Harvard PHD2000, Harvard Apparatus, Holliston, MA, USA and ProSense NE-1600, ProSense B.V., Oosterhout, Netherlands). The pH was maintained at pH 6.70 with 1 M NaOH by titration (877 Titrino Plus, Metrohm AG, Barendrecht, Netherlands) and the titration was continued for 20 min after reaching the required composition of the micelles to equilibrate the pH. In a typical experiment, approximately 3 mL 1 M NaOH was used. The total volume of the solutions was brought to 150 mL with water.

2.2.3. Ultracentrifugation and determination of apparent casein micelle hydration

Approximately 17 mL solution was equilibrated at room temperature for 1 h before ultracentrifugation at 100,000xg and 20 °C for 1 h in a Beckman Coulter Optima XE-90 ultracentrifuge (Beckman Coulter Inc., Woerden, Netherlands) equipped with a 70Ti rotor. All samples were centrifuged in duplicate. About half of each supernatant was collected and the supernatants of corresponding samples were combined. Ultracentrifugal pellets were weighed in aluminium crucibles and dried at 105 °C for approximately 48 h in a hot air oven (Binder model E 28, Binder GmbH, Tuttlingen, Germany). The apparent casein micelle hydration was calculated according to Huppertz et al. (2017) by dividing the moisture content of the pellet by the dry matter of the pellet.

2.2.4. Analysis of sedimentable casein content

The total casein content in ACM solutions and their ultracentrifugal supernatants were quantified using the Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific B.V., Breda, Netherlands) according to the corresponding protocol. A six-point calibration curve was prepared from a solution of native casein and dephosphorylated casein in a 5:5 ratio. Samples were incubated at 37 °C for 30 min in a heating block (Eppendorf ThermoMixer C, Eppendorf SE, Hamburg, Germany) and were subsequently cooled at 4 °C for 5 min. The absorbance was then measured spectrophotometrically at room temperature at a wavelength of 562 nm (Hach Lange DR6000, Hach Lange GmbH, Düsseldorf, Germany) in polystyrene cuvettes (67.742, Sarstedt AG & Co. KG, Nümbrecht, Germany). Samples were analysed in duplicate. The amount of micellar (sedimentable) casein was calculated as the difference between the total and nonsedimentable casein.

2.2.5. Quantification of cations and anions

The content of cations (calcium, phosphorus, magnesium, sodium, and potassium) of protein powders, ACM solutions, and their ultracentrifugal supernatants was analysed by inductively coupled plasma optical emission spectrometry (ICP-OES). First, samples were prepared by microwave-assisted wet digestion. Approximately 0.1 g dry material or 0.5 mL solution was weighed and 10 mL *aqua regia* (7.5 mL HCl + 2.5 mL HNO_3) and 1 mL H_2O_2 were added. Subsequently, the mixture was subjected to a temperature program in an ETHOS EASY microwave digestion system (Milestone Srl, Sorisole, Italy) according to Guimarães et al. (2021). The digested material was washed and diluted with water to a total volume of 100 mL. Samples were prepared in duplicate and analysed in triplicate with the operational conditions as specified in Guimarães et al. (2021). Elements were detected in radial view, unless stated otherwise, at the following wavelengths: Ca 393.366 nm, P 178.221 nm (axial), Mg 279.553 nm, Na 589.592 nm, K 766.490 nm.

The content of anions (chloride, phosphate, and citrate) of protein powders, ACM solutions, and their ultracentrifugal supernatants was analysed by ion chromatography (IC). Sodium caseinate and dephosphorylated sodium caseinate powders were first dissolved in water in duplicate. ACM solutions were diluted 200-fold and supernatants were diluted 500-fold in water. Solutions were then analysed on a Dionex ICS-6000 liquid chromatography system equipped with a 2 mm standard bore Dionex IonPac AS17-C column for anion analysis (Thermo Fisher Scientific B.V., Breda, Netherlands) at 30 °C with a flow rate of 0.25 mL min^{-1} and injection volume of 5 μL. Gradient elution was conducted with KOH, first set to 5 mM for 10 min, followed by a linear increase to 40 mM within 15 min, an isocratic elution at 40 mM for 6 min, and a linear decrease to 5 mM in 5 min. A conductivity detector was used for peak detection. Samples were analysed in duplicate.

All supernatant concentrations of ionic species were corrected for the excluded volume with a correction factor K calculated according to Pierre and Brule (1981). The concentration of ionic species in the micellar (sedimentable) phase was calculated by subtracting the nonsedimentable content in the supernatants from the total content in the ACM solutions. Ion speciation of organic ester phosphate in casein micelles was calculated according to White & Davies (1958).

2.2.6. Determination of calcium phosphate nanocluster size

Small-angle X-ray scattering (SAXS) measurements were performed with a laboratory SAXS instrument (Xeuss 3.0, Xenocs SAS, Grenoble, France) equipped with a GeniX 3D μ-source X-ray beam delivery system emitting the wavelength of the Cu-Kα line and a Pilatus3 R 300 K detector (Dectris AG, Baden-Daettwil, Switzerland). The sample-to-detector distance was 90 cm and the accessible q-range was 0.004 to 0.330 $Å^{-1}$. The beam size on the sample was 0.5 mm. Undiluted samples were filled into borosilicate glass capillaries of 1.5 mm in diameter (B-15–001-80, WJM-Glas/Müller GmbH, Berlin, Germany). Data collection was performed at room temperature. The raw scattering data were corrected for the background signal from simulated milk ultrafiltrate (SMUF) measured in a capillary with the same diameter and instrumental noise. SMUF was prepared according to Jenness & Koops (1962) with the modifications proposed by Dumpler et al. (2017). Isotropic 2D data were azimuthally integrated to obtain I(q)-scattering intensities versus q.

2.2.7. Particle size analysis

The size and polydispersity of casein micelles were analysed with a Malvern Zetasizer Ultra (Malvern Panalytical Ltd, Worcestershire, UK) with a He-Ne laser at a wavelength of 633 nm. Samples were diluted 100-fold in SMUF in a DTS0012 cell and measured at 25 °C with a fixed scattering angle of 173°. The refractive index of the dispersant was set at 1.33, its viscosity at 0.8872 mPa.s, and the refractive index of the casein micelles at 1.57 (Griffin & Griffin, 1985). For each sample, duplicate measurements were performed, each consisting of 5 sub-measurements. Recorded parameters were the Z-average hydrodynamic diameter and the polydispersity index, both provided by the ZS Xplorer software (version 2.3.1.4).

2.2.8. Scanning electron microscopy

Micelle morphology was investigated with scanning electron microscopy (SEM) by using a Magellan 400 microscope (FEI Company, Hillsboro, OR, USA). One drop of ACM solution was pipetted onto 12 mm poly-L-lysine glass slides (Corning Inc., Corning, NY, USA) and left to adhere for 30 min. Subsequently, the glass slides were washed twice with a 0.1 M phosphate/citrate buffer at pH 7.2 and samples were fixed with 2.5% glutaraldehyde for 1 h after removal of the buffer. Afterwards, the fixative was removed and the glass slides were washed six times with phosphate/citrate buffer. Samples were then fixed with a 1% osmium tetroxide solution for 1 h. The fixative was removed again and the slides were washed thrice with water and dehydrated using a graded ethanol series (30%, 50%, 70%, 80%, 90%, 96% EtOH all 5 min, followed by 100% EtOH twice for 10 min). Subsequently, the samples were critical point dried with CO_2 using a Leica EM CPD 300 (Leica Biosystems GmbH, Nussloch, Germany). The sputter-coated slides were

affixed to an aluminium specimen stub using carbon adhesive tabs. Mounted specimens were coated with a 12 nm tungsten layer in a Leica EM SCD 500 sputter coater. Images were taken at 100,000x magnification.

2.2.9. Rheological characterisation of rennet-induced coagulation

Rennet-induced coagulation was monitored through oscillatory rheometry according to Schenkel et al. (2011) and Kern et al. (2019). ACM solutions were adjusted to pH 5.8 and 6.3 by means of acidification below 10 °C with a 1:10 lactic acid solution in water (Kern et al., 2018) and part was left unadjusted (pH 6.7). Subsequently, 0.04% (v/w) calcium chloride was added by means of a 4% (w/v) calcium chloride solution and the samples were heated to 30 °C while stirring. Samples were then renneted by adding 0.02% (v/w) chymosin and transferred to a rheometer (MCR 302, Anton Paar, Graz, Austria) equipped with a double-gap device (DG26.7). A strain amplitude of 0.001 and a frequency of 1 Hz were applied while the temperature was controlled by a Peltier element set to 30 °C. Samples were analysed in duplicate.

2.2.10. Data analysis

Descriptive statistics were computed with Microsoft Excel 365 (version 2208). Graphs were created with OriginPro (version 2022, OriginLab Corporation, Northampton, MA, USA). Unless otherwise specified, results are expressed as the mean ± standard deviation of duplicate analyses.

3. Results

Na-CN was enzymatically dephosphorylated by means of a treatment with alkaline phosphatase and the solution was then subjected to diafiltration until all solutes were removed. Subsequently, the protein was lyophilised. This yielded a dephosphorylated Na-CN (Na-CN_{DP}). The protein content and the contents of total phosphorus and soluble inorganic phosphate were then analysed to determine the degree of dephosphorylation of the casein.

This procedure had a protein yield of 88.8% and resulted in a material with a protein content of 93.1 ± 1.8% (Table 1). The organic phosphorus attached to the phosphoserine residues of casein was calculated as the difference between the total phosphorus and the inorganic phosphorus present in the form of soluble phosphate (Law & Leaver, 1998). This calculation revealed that 10.8% of the organic phosphorus initially present in the Na-CN remained attached to the casein (Table 1). Thus, the Na-CN_{DP} was 89.2% dephosphorylated. Untreated Na-CN will be referred to as "native casein" and Na-CN_{DP} as "dephosphorylated casein" throughout this paper.

ACM were prepared from native bovine casein and the dephosphorylated casein in various ratios ranging from exclusively native to exclusively dephosphorylated casein. The level of sedimentable casein in these solutions was determined to understand the micelle-forming ability of dephosphorylated casein.

In ACM solutions prepared with only native casein, 92.0 ± 0.2% of the total casein precipitated upon ultracentrifugation (Fig. 1). This is in good agreement with the level of sedimentable casein observed for natural bovine casein micelles (Reiter et al., 2022) and artificial casein micelles (Schmidt & Poll, 1989). Decreasing the ratio of native to dephosphorylated casein linearly decreased the level of sedimentable casein, similar to the findings by Schmidt & Poll (1989). In ACM solutions prepared with less than 70% native casein, slightly more casein sedimented than the total amount of native casein present. As not all native casein sedimented in the solution with only native casein, this suggests that some dephosphorylated casein co-sedimented with the native casein. Interestingly, 10.9 ± 3.1% of the casein in the solution prepared with only dephosphorylated casein sedimented upon ultracentrifugation, whereas dephosphorylated casein did not sediment (i.e. was fully soluble) in a preliminary experiment where it was dissolved in water by stirring at 60 °C for 30 min at an equal protein concentration in the absence of calcium phosphate (data not shown).

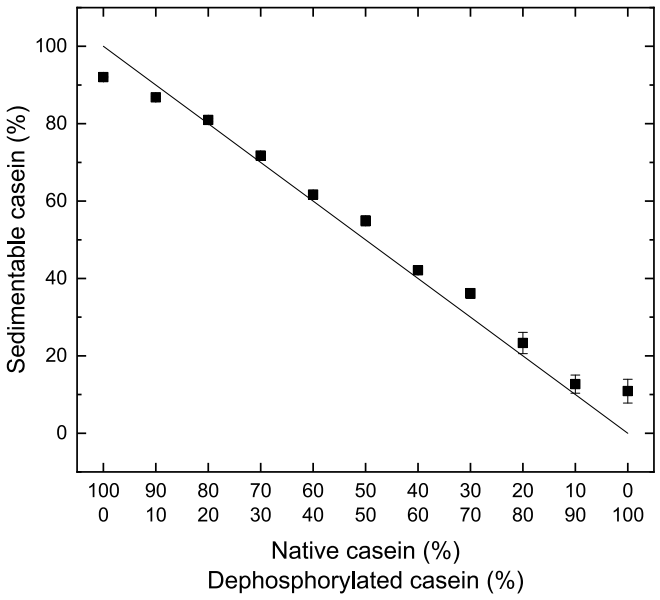

Fig. 1. Sedimentable casein as a percentage of the total casein in ACM solutions prepared with various ratios of native casein to dephosphorylated casein. The diagonal line is not a linear fit but merely serves as a guide to the eye.

SAXS measurements were performed to assess the effect of casein dephosphorylation on its ability to stabilise calcium and phosphate. Scattering intensities of ACM solutions prepared with only native casein and ACM solutions prepared with only dephosphorylated casein are shown in Fig. 2. The plateau at intermediate q-range has been related to substructures within casein micelles and the nature of these substructures has been debated in literature (Pedersen et al., 2022). The plateau at 0.07–0.10 $Å^{-1}$ for the ACM solution prepared with only native casein is dominated by the scattering contribution of protein nanoparticles and the shift of this plateau to lower q-values (around 0.03 $Å^{-1}$) upon an decreasing ratio of native to dephosphorylated casein can be explained by a dominant contribution of CaP nanoclusters (Pedersen et al., 2022). The plateau partly disappeared upon a decreasing ratio of native to dephosphorylated casein. Usually, an absence of this plateau is linked to an absence of nanoclusters, as for example upon dissolution of calcium phosphate upon acidification to pH 5.2 (Marchin et al., 2007). Alternatively, a polydisperse size distribution of the CaP nanoclusters and protein particles could have caused the gradual disappearance of the plateau. We assume that this, rather than an absence of both kinds of particles, caused the gradual disappearance of the plateau since the

Table 1
Protein content, content of total phosphorus (P_{tot}) and inorganic phosphorus (P_i), calculated content of organic phosphorus attached to casein (P_o), and the degree of casein phosphorylation of untreated Na-CN and Na-CN_{DP} treated with alkaline phosphatase.

Material	Protein content (%)	P_{tot} (mmol 100 g^{-1} casein)	P_i (mmol 100 g^{-1} casein)	P_o (mmol 100 g^{-1} casein)	Phosphorylation (%)
Na-CN	90.2 ± 0.5	26.6 ± <0.1	2.1 ± 0.4	24.5	100
Na-CN_{DP}	93.1 ± 1.8	2.8 ± <0.1	0.1 ± <0.1	2.6	10.8

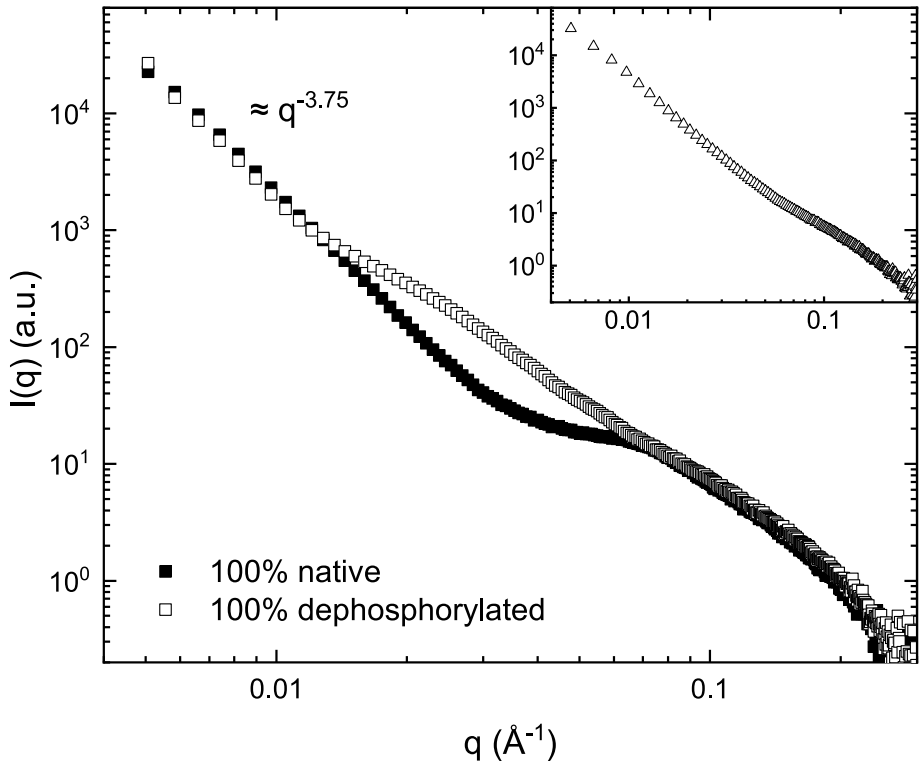

Fig. 2. Scattering intensities of ACM solutions prepared with either native casein or dephosphorylated casein. The inset graph is an example of the absence of a plateau in the curve of ACM prepared with predominantly dephosphorylated casein (20:80 is shown).

SAXS signal of the ACM solution prepared with only dephosphorylated casein again shows a well-defined plateau (presence of larger nanoclusters; Fig. 2). Moreover, the sedimentable concentrations of ionic species (Fig. 4) and the stability of the solutions against visual precipitation at storage conditions suggest that calcium and phosphate must have been present in the form of nanoclusters.

Since the plateau partly disappeared for ACM prepared with mixtures of native and dephosphorylated casein (Fig. 2 inset graph), the radii of gyration of the apparent nanoclusters in ACM prepared with 50% to 90% dephosphorylated casein could not be extracted. Nevertheless, direct application of Guinier's approximation on suitable SAXS curves showed that the size of the apparent CaP nanoclusters and protein particles gradually increased with increasing amounts of dephosphorylated casein (Fig. 3). The size of the particles in ACM prepared with only native casein (dominant contribution of protein particles) corresponds well to literature (De Kruif et al., 2012), but particles in ACM prepared with only dephosphorylated casein (dominant contribution of CaP nanoclusters) were about three times larger in diameter. However, no visual calcium phosphate crystallisation and subsequent precipitation was observed during storage for several weeks, whereas a control solution prepared without protein resulted in calcium phosphate precipitation within minutes (not shown).

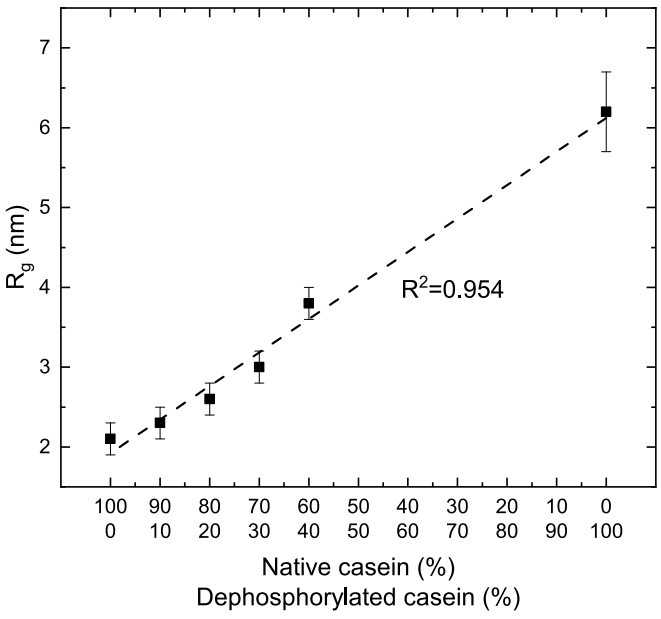

Fig. 3. Radius of gyration of calcium phosphate nanoclusters in ACM prepared with mixtures of native casein and dephosphorylated casein.

The concentrations of ionic species in the sedimentable phase were studied to assess the impact of dephosphorylated casein on the composition of CaP nanoclusters (Fig. 4). All ACM were prepared with similar total concentrations of calcium, inorganic phosphorus, magnesium, and citrate (Supplementary material A, Table S1), which were distributed over the sedimentable and nonsedimentable phases. The total and sedimentable concentrations of the ionic species correspond well to the results of Bijl et al. (2013) and the proportion of the ionic species that resided in the sedimentable phase was fairly stable in the prepared ACM. It is not surprising that the sedimentable proportion of calcium and phosphate was constant, as this is likely governed by their solubility, which was equal for all solutions since the conditions during preparation and storage (e.g. temperature, pH) did not differ. These stable concentrations of calcium and phosphate can be expected to attract constant concentrations of magnesium and citrate. Therefore, similar concentrations of the ionic species would have been insoluble and were colloidally stabilised by interaction with (dephosphorylated) casein.

Since the amount of sedimentable casein decreased upon dephosphorylation (Fig. 1), these caseins interacted with increasing amounts of ionic species (Fig. 5), similar to the results of Schmidt & Poll (1989). Fig. 5 also shows that casein mineralisation was particularly increased in ACM prepared with predominantly dephosphorylated casein, whereas

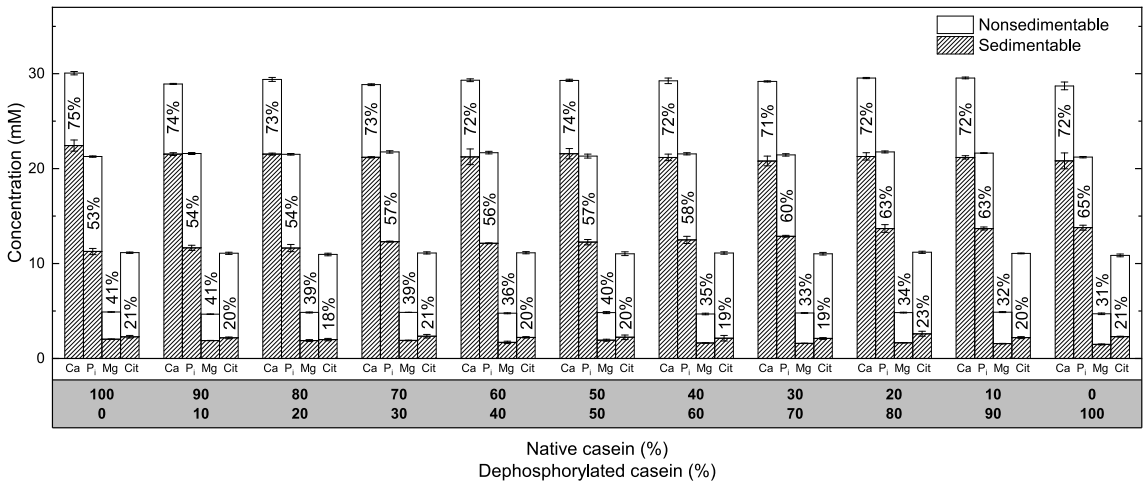

Fig. 4. Sedimentable and nonsedimentable concentrations of calcium (Ca), inorganic phosphorus (P_i), magnesium (Mg), and citrate (Cit) in the prepared ACM. The percentages indicate the sedimentable proportion of the total concentration of the ionic species.

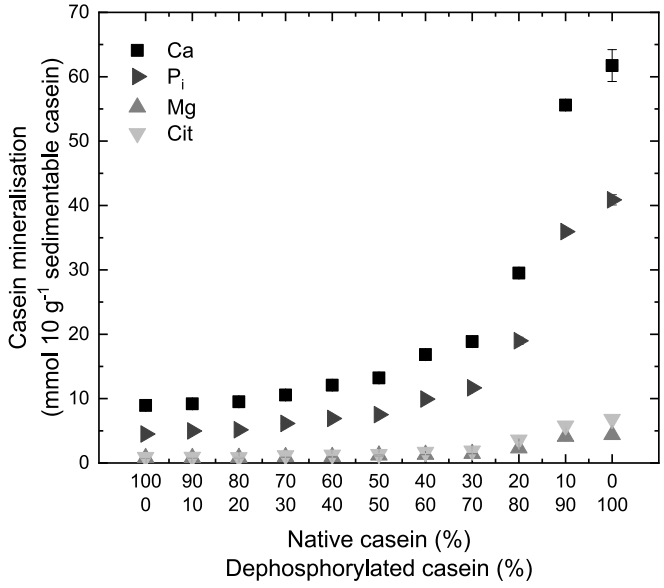

Fig. 5. Casein mineralisation expressed as the amount of calcium (Ca), inorganic phosphorus (P_i), magnesium (Mg), and citrate (Cit) per 10 g sedimentable casein.

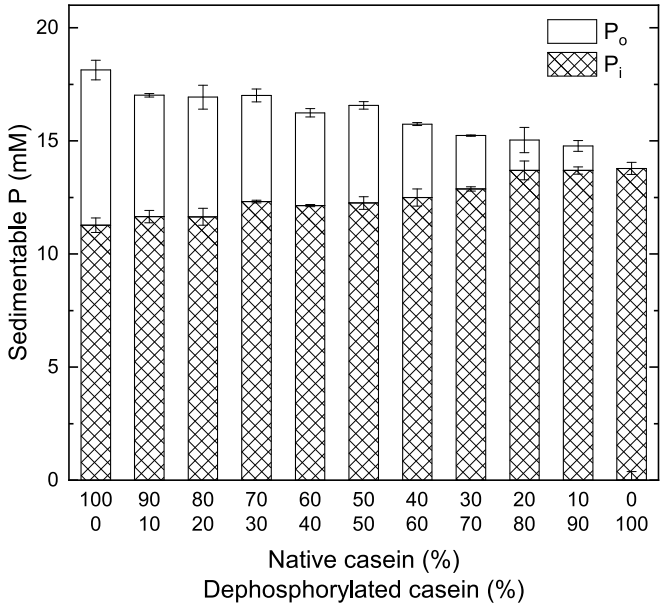

Fig. 6. Concentration of sedimentable inorganic phosphorus (P_i) and organic phosphorus (P_o).

this effect was less strong in ACM prepared with predominantly native casein.

Although the sedimentable proportions of the ionic species were relatively constant, small deviations were observed in the amount of sedimentable inorganic phosphorus, which increased upon casein dephosphorylation from 53% to 65% of the total phosphorus in the solutions (Fig. 4). This translates to a near-linear increase ($R^2 = 0.781$) from 11.3 ± 0.3 to 13.8 ± 0.3 mM (Fig. 6). Thereby, the inorganic phosphorus partially substituted the organic phosphorus in the CaP nanoclusters (which linearly ($R^2 = 0.955$) decreased from 6.9 ± 0.4 to 0.0 ± 0.4 mM as a logical consequence of the enzymatic dephosphorylation of casein) in order to maintain stoichiometry. This substitution was only partial since the overall sedimentable phosphorus concentration decreased. The subsequent lower concentration of anions in the sedimentable phase then attracted less counterions, such as magnesium, the sedimentable concentration of which gradually decreased from 41% to 31% of the total magnesium in ACM with increasing amounts of dephosphorylated casein (Fig. 4). The concentration of sedimentable calcium was unaffected.

The prepared ACM were analysed by dynamic light scattering to determine the ability of dephosphorylated casein to assemble into micelle-like particles. The average diameter of the particles first decreased for ACM prepared with 100% to 80% native casein, then stabilised for ACM prepared with 80–60% native casein and then increased again when prepared with 50–0% native casein (Fig. 7). The size of ACM prepared with increased amounts of dephosphorylated casein proved difficult to measure accurately with dynamic light scattering, as indicated by the large error bars. However, the polydispersity index showed no considerable variety and the size distributions remained monomodal (data not shown). Inaccuracies could have arisen from the morphology of the particles, which became increasingly irregular upon preparation with increasing dephosphorylated casein (Fig. 8). Dynamic light scattering typically predicts high standard deviations for non-spherical particles and detects the size of such shapes with decreased accuracy (Arenas-Guerrero et al., 2018).

ACM were further characterised based on their apparent hydration (Fig. 9). The apparent hydration of the sedimentable protein was

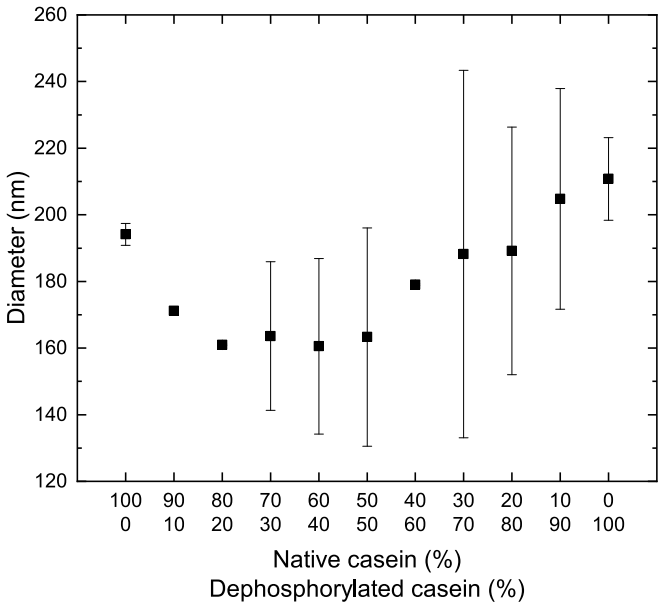

Fig. 7. Diameter of particles prepared with various ratios of native casein to dephosphorylated casein.

initially stable upon a decreasing ratio of native to dephosphorylated casein, after which it decreased to 1.95 ± 0.01 g water g^{-1} protein for solutions prepared with 10% native casein and 90% dephosphorylated casein. ACM prepared with only dephosphorylated casein showed a similar apparent hydration of 1.98 ± 0.03 g water g^{-1} protein.

The rennet-induced coagulation of the prepared ACM was monitored at three different pH values. The development of the storage moduli (as a measure of curd firmness) during renneting of ACM prepared with only native casein and ACM prepared with 50% native casein and 50% dephosphorylated casein are shown as examples (Fig. 10A and B). For both systems, renneting at lower pH values decreased the time until the onset of coagulation to a minimum of about 4 min at pH 5.8. Generally, coagulation proceeds faster at reduced pH values due to a reduction of the electrostatic repulsion between caseins and accelerated rennet activity (Lucey et al., 2003). In parallel, micellar calcium phosphate is partly solubilised at pH < 6.0, which causes an increase in the electrostatic repulsion between caseins due to the exposed negatively charged phosphoserine residues and a decrease in the number of nanocluster crosslinks (Lucey et al., 2003). As a result, decreasing the pH from 6.7 to 6.3 increased the maximum recorded curd firmness of ACM prepared with only native casein from 67.8 ± 0.4 to 133.0 ± 0.3 Pa, whereas a further decrease of the pH value to 5.8 reduced it to 98.8 ± 6.3 Pa (Fig. 10A).

These measurements were repeated for ACM prepared with mixtures of native and dephosphorylated casein and the maximum storage modulus during an hour of renneting was recorded (Fig. 11). ACM prepared with increasing amounts of dephosphorylated casein generally yielded curds with decreased firmness after an hour of incubation with rennet. At pH 6.3, this decrease was linear ($R^2 = 0.940$) and solutions at pH 6.7 showed a linear decrease until the ACM prepared with 70% native and 30% dephosphorylated casein ($R^2 = 0.994$), after which the maximum storage modulus was virtually zero. The maximum storage modulus attained at pH 5.8 first decreased sharply from 98.8 ± 6.3 to 44.3 ± 8.1 Pa, after which it stagnated (and even increased slightly) for ACM prepared with 90% to 50% native casein before further decreasing to 5.5 ± 0.1 Pa for ACM prepared with only dephosphorylated casein.

To assess the influence of the dephosphorylated casein on the gelation, ACM prepared with only native casein were diluted in SMUF (to correspond with the concentrations of native casein in the mixtures) to simulate the micellar phase of ACM prepared with mixtures of native and dephosphorylated casein. ACM prepared with only native casein yielded a softer *coagulum* upon renneting at pH 6.7 when diluted to 90% native casein but a firmer *coagulum* when diluted to 70% and 50% in comparison to ACM prepared with these amounts of native casein and complementary amounts of dephosphorylated casein (Fig. 12).

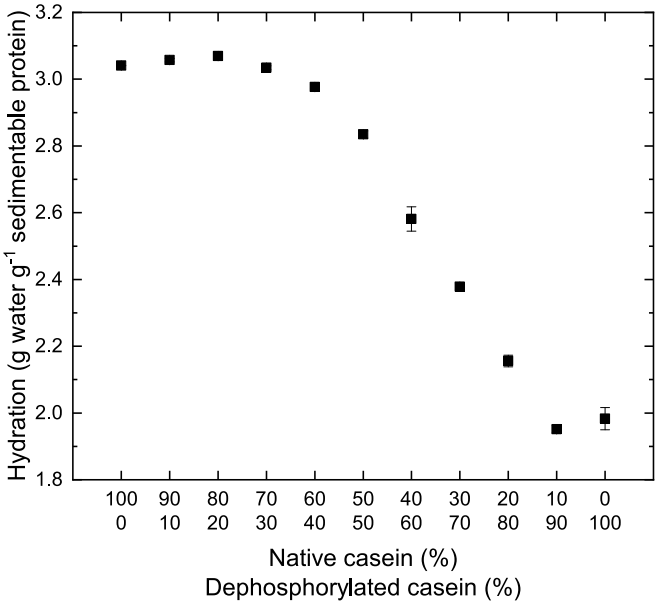

Fig. 9. Apparent hydration of ACM prepared with various ratios of native casein to dephosphorylated casein.

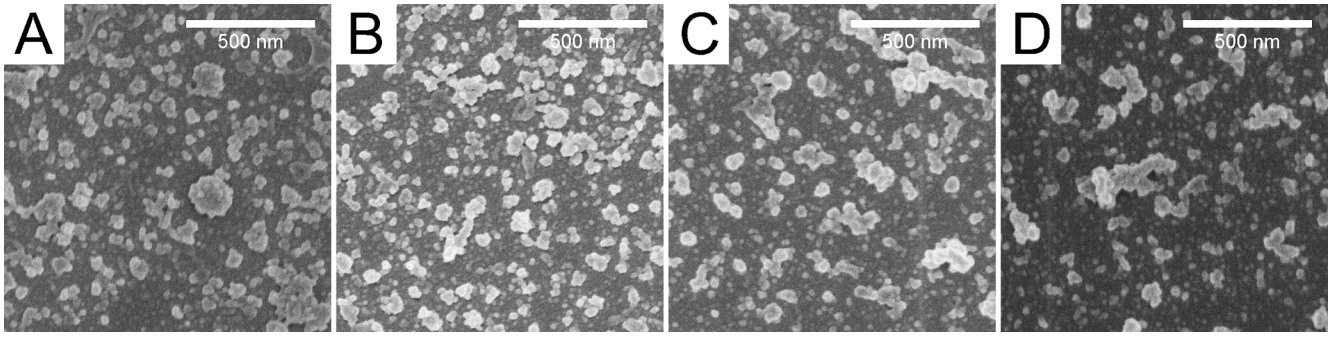

Fig. 8. Scanning electron microscopy images of ACM prepared with (A) 100%, (B) 70%, (C) 30%, and (D) 0% native casein and complementary percentages of dephosphorylated casein. Scale bar = 500 nm.

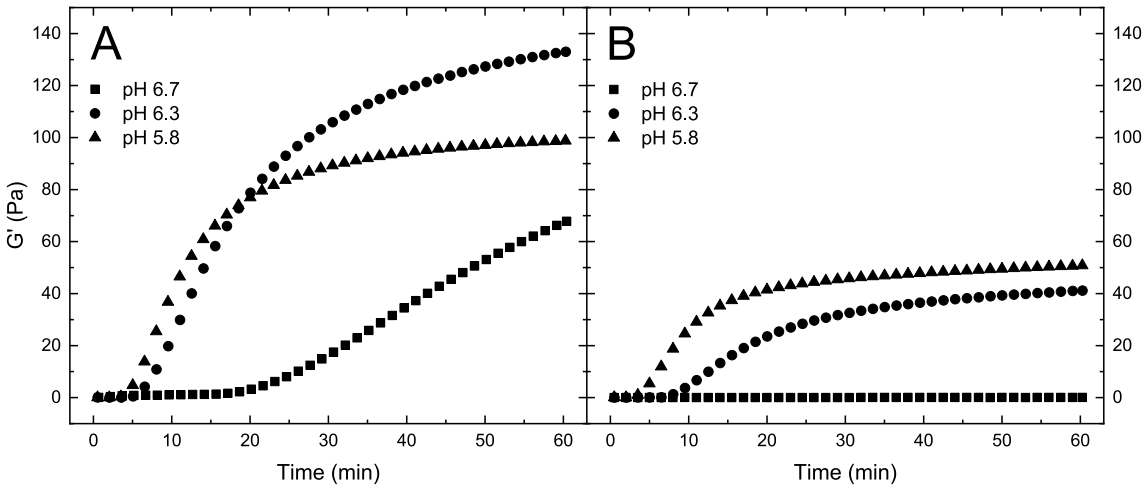

Fig. 10. Development of the storage modulus G' at three different pH levels during renneting of (A) ACM prepared with only native casein and (B) ACM prepared with 50% native casein and 50% dephosphorylated casein.

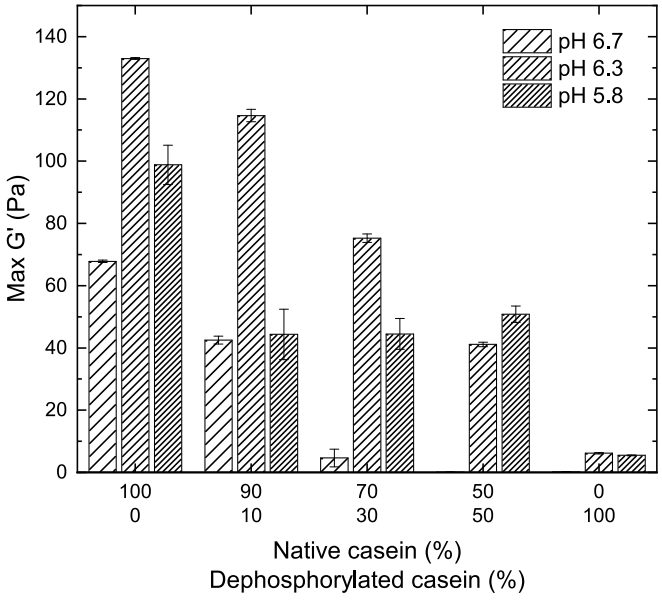

Fig. 11. Maximum recorded storage modulus (G') during one hour of renneting at different pH levels.

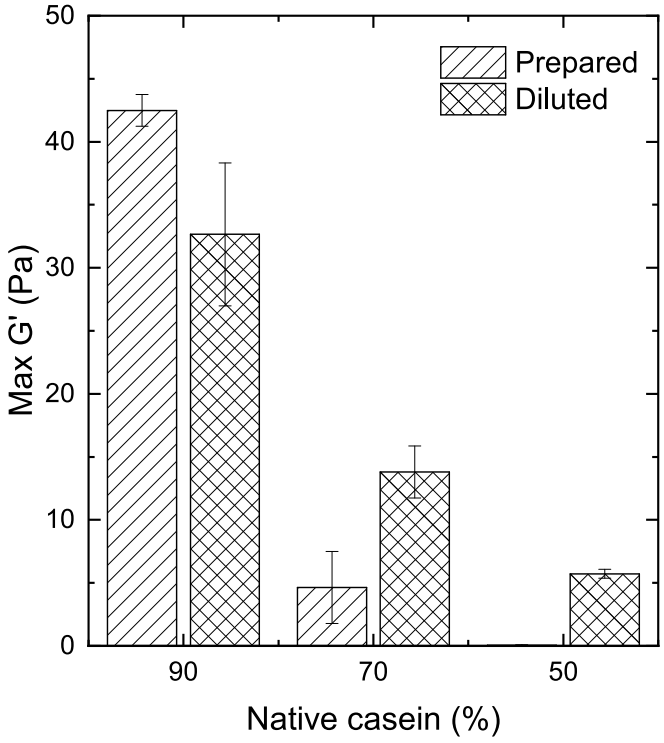

Fig. 12. Maximum recorded storage modulus (G') during one hour of renneting at pH 6.7 of ACM prepared with decreasing amounts of native casein (and increasing amounts of dephosphorylated casein) and ACM prepared with only native casein diluted in SMUF to various concentrations.

4. Discussion

4.1. Micelle formation of dephosphorylated casein

A large proportion of the casein in ACM solutions prepared with predominantly native casein (i.e. not treated with alkaline phosphatase) sedimented upon ultracentrifugation (Fig. 1) and this casein formed highly hydrated (Fig. 9), spherical particles of >150 nm in diameter (Fig. 7, Fig. 8A), which stabilised high concentrations of ionic species in the form of apparent CaP nanoclusters (Fig. 3, Fig. 4). It is therefore reasonable to consider these particles artificial casein micelles. The particles in solutions prepared with predominantly dephosphorylated casein, were hydrated less, shaped more irregularly (Fig. 8D), and consisted of less casein, although these caseins stabilised similar concentrations of ionic species that formed bigger apparent nanoclusters. These particles have therefore been referred to as calcium phosphate particles colloidally stabilised by the adsorption of casein, rather than true casein micelles (Schmidt & Poll, 1989). However, the apparent CaP nanoclusters in these samples were not larger than about 6 nm (Fig. 3), whereas we observed protein particles of over 200 nm in diameter. This indicates that the casein-stabilised CaP nanoclusters assembled into larger particles, similar to casein micelles. Since the term 'casein micelle' is notoriously ill-defined and used to refer to a variety of particles in colloidal dispersions (Fox & Brodkorb, 2008), we will also refer to the particles formed in solutions prepared with predominantly dephosphorylated casein as 'artificial casein micelles' for consistency.

Upon a decreasing ratio of native to dephosphorylated casein, the native casein likely continued to form micelles, whereas the ability of dephosphorylated casein to assemble into micelles is significantly impaired by the loss of their phosphate groups and their subsequent inability to colloidally stabilise CaP nanoclusters through these groups.

As a result, the amount of sedimentable casein decreased linearly (Fig. 1) and it can be assumed that the number of micelles also decreased. This emphasises that the phosphate centres of native casein are the primary sites of interaction with CaP nanoclusters and are crucial for the formation of casein micelles (Aoki et al., 1992). However, at least part of the dephosphorylated casein seems to have been incorporated into the ACM through interaction with other caseins or with CaP nanoclusters, as the amount of sedimentable casein exceeded the amount of native casein present for ACM prepared with less than 70% native casein. Dephosphorylated κ-casein likely continued to contribute to the assembly into micelles since native κ-casein contains only one phosphoserine residue and no phosphate centres (De Kruif & Holt, 2003). Instead, it interacts with and limits the size of casein micelles through hydrophobic interactions (Horne, 1998) and this functionality is retained upon dephosphorylation (Pepper & Thompson, 1963). As a result, the increasing amount of functional κ-casein relative to the amount of calcium-sensitive casein must have increased, which explains the initial decrease in size of ACM prepared with less than 30% dephosphorylated casein (Fig. 7). Further increasing the amount of dephosphorylated casein then increased the size of ACM again due to a second size-affecting phenomenon; the volume of CaP nanoclusters.

The internal structure of the ACM was analysed with SAXS (Fig. 2; De Kruif et al., 2012) and while SAXS is no unequivocal method to determine the size of the nanoclusters, there is evidence that the cluster size increased linearly from approximately 2 to 6 nm upon a decreasing ratio of native to dephosphorylated casein (Fig. 3), while their composition was largely unaffected (Fig. 4). On the assumption that these are CaP nanoclusters, they must have occupied a larger part of the fewer remaining micelles and thus increased their overall volume considerably. This could explain the increase in micelle size in the presence of the increasing surplus of functional κ-casein for ACM with more than 50% dephosphorylated casein (Fig. 7). Thus, the size decrease due to the increasing amount of functional κ-casein relative to the amount of calcium-sensitive casein was counteracted in ACM with higher amounts of dephosphorylated casein due to the increasing number of larger CaP nanoclusters within a single micelle, as illustrated in Fig. 13. This effect became increasingly dominant for ACM prepared with more than 40% dephosphorylated casein, which corresponds to the gradually steeper increase in the degree of casein mineralisation in these ACM (Fig. 5).

Furthermore, it is likely that part of the dephosphorylated casein stabilised CaP nanoclusters from further growth since about 10% of the dephosphorylated casein in the solution prepared with only dephosphorylated casein co-sedimented with the CaP nanoclusters (Fig. 1), whereas no sedimentation of dephosphorylated casein was observed in absence of calcium and phosphate (data not shown). Such interactions might be established through the residual phosphoserine residues on the dephosphorylated casein after enzymatic dephosphorylation, although it is unlikely that intact phosphate centres were present. Alternatively, dephosphorylated casein could have contributed to stabilising the nanoclusters by adsorbing to their surface. A variety of biomacromolecules as well as negatively charged synthetic polyelectrolytes or unphosphorylated protein sequences can stabilise amorphous calcium phosphate against crystallisation at concentrations of about 1 mM (Bar-Yosef Ofir et al., 2004; Gelli et al., 2019). Caseins are natural polyelectrolytes that carry a considerable number of negatively charged amino acids at neutral pH (even after dephosphorylation) and were present at around 1 mM in the ACM solutions. Thus, it is reasonable to assume that negatively charged sequences of dephosphorylated casein exerted a similar stabilising effect on the amorphous CaP nanoclusters. These casein-stabilised nanoclusters formed irregularly-shaped particles (Fig. 8D, Fig. 13), where the irregular shape may have originated from the altered weak interactions (e.g. electrostatic, hydrophobic) between caseins as a consequence of their dephosphorylation and the decreased amount of κ-casein binding sites (i.e. calcium-sensitive caseins).

The increasing volume of the apparent CaP nanoclusters in the ACM also affected their hydration. ACM prepared with predominantly native casein comprise an extensive colloidal casein network that interacts strongly with water. It can be expected that such a protein network is diminished in the ACM prepared with predominantly dephosphorylated casein, due to the increasing volume of larger CaP nanoclusters and decreasing amount of micellar casein. As a result, the hydration of the ACM decreased upon a decreasing ratio of native to dephosphorylated casein (Fig. 9). This trend became particularly apparent in ACM solutions prepared with over 40% dephosphorylated casein, because the degree of casein mineralisation was increasingly high at high amounts of dephosphorylated casein (Fig. 5). Since the amount of sedimentable casein decreased upon casein dephosphorylation (Fig. 1) and the hydration of this smaller sedimentable casein fraction decreased as well, this means that the volume fraction of ACM in the solutions decreased by a factor of about 12 from ACM prepared with only native casein to ACM prepared with only dephosphorylated casein.

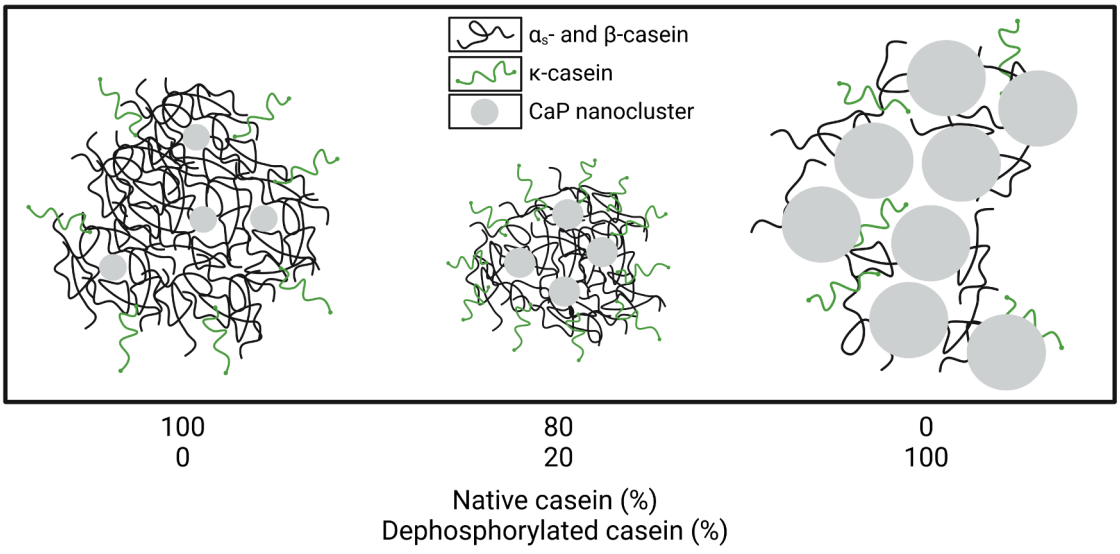

Fig. 13. Schematic depiction of the structure of ACM prepared with (a mixture of) native casein and dephosphorylated casein. Created with BioRender.com.

4.2. Rennet-induced coagulation

The decreased volume fraction and the different properties of the ACM also affected the rennet coagulation. Generally, dephosphorylated casein did not contribute to the formation of a gel network upon rennet-induced coagulation, as evident from the decreasing storage moduli upon increasing amounts of dephosphorylated casein (Fig. 11). In fact, dephosphorylated casein actively obstructed the formation of a coherent gel network upon rennet-induced coagulation (Fig. 12), as the moduli are lower with dephosphorylated casein present at equal concentrations of native casein. This effect can partly be related to the decreased hydration of ACM prepared with mixtures of native and dephosphorylated casein (Fig. 9). This decreased the volume fraction of these ACM compared to the diluted ACM prepared with only native casein, which resulted in lower maximum storage moduli. Additionally, the increased concentrations of nonsedimentable casein in the serum at higher amounts of dephosphorylated casein (Fig. 1) could have had an effect. It has already been found that increasing concentrations of whey protein in the serum act as an inert filler increasingly inhibiting the enzymatic action of chymosin and presenting a physical barrier for casein micelle aggregation (Gamlath et al., 2018). It is reasonable to assume that dephosphorylated casein in the serum phase exerts a similar effect, thereby decreasing the maximum storage modulus. For ACM prepared with 90% native casein and 10% dephosphorylated casein, the protein concentration in the serum was only slightly higher, which did not impair the firmness of the curd to a large extent. Instead, the corresponding diluted ACM from only native casein formed a weaker gel in this instance. This could be related to the size of the micelles in both systems, where it is assumed that dilution of the artificial casein micelles from only native casein in SMUF did not influence their size. In that case, the micelles prepared with 90% dephosphorylated and 10% native casein were considerably smaller than the diluted micelles prepared with only native casein (Fig. 7). Smaller casein micelles have previously been linked to the formation of a firmer and more compact gel network upon rennet coagulation (Niki & Arima, 1984).

5. Conclusions

This study reiterated that the ability of calcium-sensitive caseins to assemble into micelles is impaired by the loss of their phosphate groups. In contrast, κ-casein retained its functionality upon dephosphorylation. Therefore, the amount of functional κ-casein relative to the amount of calcium-sensitive casein increased, which decreased the size of the artificial casein micelles prepared with low amounts of dephosphorylated casein. The concentrations of sedimentable ionic species were unaffected by casein dephosphorylation, which resulted in increasing degrees of casein mineralisation and a threefold increase in the size of calcium phosphate nanoclusters in artificial casein micelles prepared with increasing amounts of dephosphorylated casein. As a result, artificial casein micelles prepared with predominantly dephosphorylated casein increased in size and were decreasingly hydrated. Moreover, these micelles showed poor rennet coagulation behaviour, where non-micellar dephosphorylated casein disrupted the formation of a coherent gel network. Taken together, these results demonstrate the significance of casein phosphorylation for the structure and integrity of casein micelles and show that dephosphorylated casein do not contribute towards the formation of hybrid micelles from native casein and dephosphorylated casein. This emphasises that post-translational phosphorylation of recombinant casein is essential to engineer artificial casein micelles for future food applications that build upon the casein micelle structure.

CRediT authorship contribution statement

Laurens J. Antuma: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Isabell Steiner:** Investigation. **Vasil M. Garamus:** Methodology, Investigation, Formal analysis, Writing – original draft. **Remko M. Boom:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Julia K. Keppler:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

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.

Acknowledgements

The work for this publication has been undertaken as part of the GOUDA project, which has received funding from the European Union's Horizon 2020 research and innovation programme through Eurostars (E! 114377). The authors would like to thank Helena Braitmaier from the University of Hohenheim for sharing her expertise on the rheological characterisation of rennet coagulation, Sandra Wilde, Thomas Schubert, and Florian Hamann from Formo for their cooperation with the dephosphorylation of casein and the determination of the proteolytic activity of alkaline phosphatase, Pieter Gremmen at the department of Environmental Technology of Wageningen University & Research for his generous assistance with the quantification of ions, and Marcel Giesbers and Jelmer Vroom of the Wageningen Electron Microscopy Centre for their help with scanning electron microscopy. The graphical abstract was created with BioRender.com.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113315.

References

Aoki, T., Umeda, T., & Kako, Y. (1992). The least number of phosphate groups for crosslinking of casein by colloidal calcium phosphate. *Journal of Dairy Science, 75*(4), 971–975. https://doi.org/10.3168/jds.S0022-0302(92)77838-2

Arenas-Guerrero, P., Delgado, Á. v., Donovan, K. J., Scott, K., Bellini, T., Mantegazza, F., & Jiménez, M. L. (2018). Determination of the size distribution of non-spherical nanoparticles by electric birefringence-based methods. *Scientific Reports*, *8*(1), 9502. https://doi.org/10.1038/s41598-018-27840-0.

Banovic, M., Barone, A. M., Asioli, D., & Grasso, S. (2022). Enabling sustainable plant-forward transition: European consumer attitudes and intention to buy hybrid products. *Food Quality and Preference, 96*, Article 104440. https://doi.org/10.1016/j.foodqual.2021.104440

Bar-Yosef Ofir, P., Govrin-Lippman, R., Garti, N., & Füredi-Milhofer, H. (2004). The influence of polyelectrolytes on the formation and phase transformation of amorphous calcium phosphate. *Crystal Growth & Design, 4*(1), 177–183. https://doi.org/10.1021/cg034148g

Bijl, E., van Valenberg, H. J. F., Huppertz, T., & van Hooijdonk, A. C. M. (2013). Protein, casein, and micellar salts in milk: Current content and historical perspectives. *Journal of Dairy Science, 96*(9), 5455–5464. https://doi.org/10.3168/jds.2012-6497

Bingham, E. W., Farrell, H. M., & Carroll, R. J. (1972). Properties of dephosphorylated α_{s1}-casein. Precipitation by calcium ions and micelle formation. *Biochemistry, 11*(13), 2450–2454. https://doi.org/10.1021/bi00763a010

Cohen, P. (2002). The origins of protein phosphorylation. *Nature Cell Biology, 4*(5), E127–E130. https://doi.org/10.1038/ncb0502-e127

Dalgleish, D. G. (1993). The enzymatic coagulation of milk. In P. F. Fox (Ed.), *Cheese: Chemistry, Physics and Microbiology* ((2nd ed.,, pp. 69–100). US: Springer. https://doi.org/10.1007/978-1-4615-2650-6_3.

Dumpler, J., Kieferle, I., Wohlschläger, H., & Kulozik, U. (2017). Milk ultrafiltrate analysis by ion chromatography and calcium activity for SMUF preparation for different scientific purposes and prediction of its supersaturation. *International Dairy Journal, 68*, 60–69. https://doi.org/10.1016/j.idairyj.2016.12.009

Farrell, H. M., Jiménez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hicks, C. L., Hollar, C. M., Ng-Kwai-Hang, K. F., & Swaisgood, H. E. (2004). Nomenclature of the proteins of cows' milk—Sixth revision. *Journal of Dairy Science, 87*(6), 1641–1674. https://doi.org/10.3168/jds.S0022-0302(04)73319-6

Fox, P. F., & Brodkorb, A. (2008). The casein micelle: Historical aspects, current concepts and significance. *International Dairy Journal, 18*(7), 677–684. https://doi.org/10.1016/j.idairyj.2008.03.002

Gamlath, C. J., Leong, T. S. H., Ashokkumar, M., & Martin, G. J. O. (2018). The inhibitory roles of native whey protein on the rennet gelation of bovine milk. *Food Chemistry, 244*, 36–43. https://doi.org/10.1016/j.foodchem.2017.10.029

Gaucheron, F. (2005). The minerals of milk. *Reproduction Nutrition Development, 45*(4), 473–483. https://doi.org/10.1051/rnd:2005030

Gelli, R., Ridi, F., & Baglioni, P. (2019). The importance of being amorphous: Calcium and magnesium phosphates in the human body. *Advances in Colloid and Interface Science, 269*, 219–235. https://doi.org/10.1016/j.cis.2019.04.011

Griffin, M. C. A., & Griffin, W. G. (1985). A simple turbidimetric method for the determination of the refractive index of large colloidal particles applied to casein micelles. *Journal of Colloid And Interface Science, 104*(2), 409–415. https://doi.org/10.1016/0021-9797(85)90049-9

Guimaräes, B. O., Gremmen, P., Wijffels, R. H., Barbosa, M. J., & D'Adamo, S. (2021). Effect of ammonium formate washing on the elemental composition determination in *Nannochloropsis oceanica*. *Aquaculture, 538*, Article 736526. https://doi.org/10.1016/j.aquaculture.2021.736526

Hettinga, K., & Bijl, E. (2022). Can recombinant milk proteins replace those produced by animals? *Current Opinion in Biotechnology, 75*, Article 102690. https://doi.org/10.1016/j.copbio.2022.102690

Holt, C. (1997). The milk salts and their interaction with casein. In P. F. Fox (Ed.), *Advanced Dairy Chemistry: Vol. 3. Lactose, water, salts and vitamins* (2nd ed., pp. 233–256). Springer US. https://doi.org/10.1007/978-1-4757-4409-5_6.

Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. *Journal of Dairy Science, 96*(10), 6127–6146. https://doi.org/10.3168/jds.2013-6831

Horne, D. S. (1998). Casein interactions: Casting light on the black boxes, the structure in dairy products. *International Dairy Journal, 8*(3), 171–177. https://doi.org/10.1016/S0958-6946(98)00040-5

Huppertz, T., Gazi, I., Luyten, H., Nieuwenhuijse, H., Alting, A., & Schokker, E. (2017). Hydration of casein micelles and caseinates: Implications for casein micelle structure. *International Dairy Journal, 74*, 1–11. https://doi.org/10.1016/j.idairyj.2017.03.006

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

Jia, B., & Jeon, C. O. (2016). High-throughput recombinant protein expression in *Escherichia coli*: Current status and future perspectives. *Open Biology, 6*(8), Article 160196. https://doi.org/10.1098/rsob.160196

Kalidas, C., Joshi, L., & Batt, C. (2001). Characterization of glycosylated variants of β-lactoglobulin expressed in *Pichia pastoris*. *Protein Engineering, Design and Selection, 14*(3), 201–207. https://doi.org/10.1093/protein/14.3.201

Keppler, J. K., Heyse, A., Scheidler, E., Uttinger, M. J., Fitzner, L., Jandt, U., … Biedendieck, R. (2021). Towards recombinantly produced milk proteins: Physicochemical and emulsifying properties of engineered whey protein beta-lactoglobulin variants. *Food Hydrocolloids, 110*, Article 106132. https://doi.org/10.1016/j.foodhyd.2020.106132

Kern, C., Stefan, T., & Hinrichs, J. (2019). Multiple linear regression modeling: Prediction of cheese curd dry matter during curd treatment. *Food Research International, 121*, 471–478. https://doi.org/10.1016/j.foodres.2018.11.061

Kern, C., Weiss, J., & Hinrichs, J. (2018). Additive layer manufacturing of semi-hard model cheese: Effect of calcium levels on thermo-rheological properties and shear behavior. *Journal of Food Engineering, 235*, 89–97. https://doi.org/10.1016/j.jfoodeng.2018.04.029

de Kruif, C. G., & Holt, C. (2003). Casein micelle structure, functions and interactions. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry: Vol. 1. Proteins* (3rd ed., pp. 233–276). Springer US. https://doi.org/10.1007/978-1-4419-8602-3_5.

de Kruif, C. G., Huppertz, T., Urban, V. S., & Petukhov, A. V. (2012). Casein micelles and their internal structure. *Advances in Colloid and Interface Science, 171–172*, 36–52. https://doi.org/10.1016/j.cis.2012.01.002

Law, A. J. R., & Leaver, J. (1998). Effects of acidification and storage of milk on dissociation of bovine casein micelles. *Journal of Agricultural and Food Chemistry, 46*(12), 5008–5016. https://doi.org/10.1021/jf980748p

Lucey, J. A., Johnson, M. E., & Horne, D. S. (2003). Invited review: Perspectives on the basis of the rheology and texture properties of cheese. *Journal of Dairy Science, 86*(9), 2725–2743. https://doi.org/10.3168/jds.S0022-0302(03)73869-7

Marchin, S., Putaux, J.-L., Pignon, F., & Léonil, J. (2007). Effects of the environmental factors on the casein micelle structure studied by cryo transmission electron microscopy and small-angle x-ray scattering/ultrasmall-angle x-ray scattering. *The Journal of Chemical Physics, 126*(4), Article 045101. https://doi.org/10.1063/1.2409933

Niki, R., & Arima, S. (1984). Effects of size of casein micelle on firmness of rennet curd. *Japanese Journal of Zootechnical Science, 55*(6), 409–415.

Pedersen, J. S., Møller, T. L., Raak, N., & Corredig, M. (2022). A model on an absolute scale for the small-angle X-ray scattering from bovine casein micelles. *Soft Matter, 18*(45), 8613–8625. https://doi.org/10.1039/D2SM00724J

Pepper, L., & Thompson, M. P. (1963). Dephosphorylation of α_s- and κ-caseins and its effect on micelle stability in the κ-α_s-casein system. *Journal of Dairy Science, 46*(8), 764–767. https://doi.org/10.3168/jds.S0022-0302(63)89145-6

Pierre, A., & Brule, G. (1981). Mineral and protein equilibria between the colloidal and soluble phases of milk at low temperature. *Journal of Dairy Research, 48*(3), 417–428. https://doi.org/10.1017/S0022029900021890

Reiter, M., Reitmaier, M., & Kulozik, U. (2022). Compositional changes of casein micelles induced by calcium or chelatant addition at threefold and natural casein concentration. *International Dairy Journal, 130*, Article 105365. https://doi.org/10.1016/j.idairyj.2022.105365

Saito, A., Usui, M., Song, Y., Azakami, H., & Kato, A. (2002). Secretion of glycosylated α-lactalbumin in yeast *Pichia pastoris*. *Journal of Biochemistry, 132*(1), 77–82. https://doi.org/10.1093/oxfordjournals.jbchem.a003202

Schenkel, P., Samudrala, R., & Hinrichs, J. (2011). Fat-reduced semi-hard cheese enriched with a microparticulated whey protein concentrate: Impact on cheese-making properties and rheological characteristics. *Milchwissenschaft, 66*(1), 43–47.

Schmidt, D. G., Koops, J., & Westerbeek, D. (1977). Properties of artificial casein micelles. 1. Preparation, size distribution and composition. *Netherlands Milk and Dairy Journal, 31*(4), 328–341.

Schmidt, D. G., & Poll, J. K. (1989). Properties of artificial casein micelles. 4. Influence of dephosphorylation and phosphorylation of the casein. *Netherlands Milk and Dairy Journal, 43*, 53–62.

Tubb, C., & Seba, T. (2021). Rethinking food and agriculture 2020–2030: The second domestication of plants and animals, the disruption of the cow, and the collapse of industrial livestock farming. *Industrial Biotechnology, 17*(2), 57–72. https://doi.org/10.1089/ind.2021.29240.ctu

White, J. C. D., & Davies, D. T. (1958). The relation between the chemical composition of milk and the stability of the caseinate complex: I. General introduction, description of samples, methods and chemical composition of samples. *Journal of Dairy Research, 25*(2), 236–255. https://doi.org/10.1017/S0022029900009249

Yamauchi, K., Takemoto, S., & Tsugo, T. (1967). Calcium-binding property of dephosphorylated caseins. *Agricultural and Biological Chemistry, 31*(1), 54–63. https://doi.org/10.1080/00021369.1967.10858770