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Invited review: Modeling milk stability

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ABSTRACT

Novel insights into the stability of milk and milk products during storage and processing result from describing caseins near neutral pH as hydrophilic, intrinsically disordered, proteins. Casein solubility is strongly influenced by pH and multivalent ion binding. Solubility is high at neutral pH or above but decreases as casein net charge approaches zero, allowing a condensed casein phase or gel to form then increases at lower pH. Of particular importance for casein micelle stability near neutral pH is the proportion of free caseins in the micelle (i.e., caseins not bound directly to nanoclusters of calcium phosphate). Free caseins are more soluble and better able to act as molecular chaperones (to prevent casein and whey protein aggregation) than bound caseins. Some free caseins are highly phosphorylated and can also act as mineral chaperones to inhibit the growth of calcium phosphate phases and prevent mineralized deposits from forming on membranes or heat exchangers. Thus, casein micelle stability is reduced when free caseins bind to amyloid fibrils, destabilized whey proteins or calcium phosphate. The multivalent-binding model of the casein micelle quantitatively describes these and other factors affecting the stability of milk and milk protein products during manufacture and storage.
Keywords: Casein micelle, calcium phosphate nanocluster, amyloid fibril, molecular chaperone, amorphous aggregation, milk protein stability

INTRODUCTION AND OVERVIEW

Milk contains high concentrations of amyloid fibril-forming proteins, and concentrations of calcium and phosphate far in excess of the solubility of di- and tri-calcium phosphate (CaP) salts. Nevertheless, it is a highly stable biofluid under physiological conditions. It may be stored in the mammary gland for days, weeks or even months, depending on the reproductive strategy of the species, without causing pathological calcification or amyloidosis of the gland (Holt and Hukins, 1991, Holt and Carver, 2012). However, in the acidic conditions of the stomach (pH 4–5), the casein fraction readily forms a gel or a more condensed phase, augmented by the proteolytic activity of gastric proteinases such as pepsin or chymosin.

Traditionally, the casein micelle has been described as a hydrophobic colloid. However, caseins in the micelle are highly hydrated proteins with a flexible conformation, which is difficult to reconcile with this description. In reviews and original publications over the last 3 decades, an increasing number of research groups have applied the rapidly developing science of hydrophilic, intrinsically disordered proteins (IDPs) to casein structure and interactions (Holt and Sawyer, 1993, Farrell et al., 2003, Morgan et al., 2005, Thorn et al., 2005, Farrell et al., 2006, Léonil et al., 2008, Yong and Foegeding, 2010, Holt et al., 2013, Librizzi et al., 2014, Ghahghaei and Shahraki, 2015, Pan and Zhong, 2015, Raynes et al., 2015, Redwan et al., 2015, Mirdha and Chakraborty, 2019, Sanders et al., 2020, Wang et al., 2020b, Bahraminejad et al., 2022, Hewa Nadugala et al., 2023, Skibsted, 2023, Carver and Holt, 2024). In this review, the trend is continued with the application of such modern concepts to help explain milk stability.

Caseins share many compositional, structural, and functional properties with other IDPs, as reviewed by (Carver and Holt, 2024). They can form amyloid fibrils or act as molecular chaperones to inhibit formation of both amyloid fibrils and amorphous protein aggregates. Caseins are highly soluble at neutral pH and are relatively hydrophilic when measured by any reliable scale of amino acid hydrophobicity. They do not have any long sub-sequences of hydrophobic residues. They adopt an open, highly hydrated, and dynamic ensemble of conformations with a preponderance of the poly-L-proline type-II secondary structure.

Interactions of IDPs that do not cause the proteins to fold into a compact conformation are multivalent. They arise from at least 2, and usually more, weak dynamic and reversible interactions, mediated through short, linear sequence motifs (SLiMs). The SLiMs in IDPs interact through dipolar, electrostatic, π-bonding, and

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hydrophobic interactions. The SLiMs identified so far in caseins (Qi et al., 2001, Carver and Holt, 2019, Holt et al., 2019) are (i) CaP-SLiMs: phosphorylated short sequences that bind directly to the CaP nanoclusters, (ii) basic-SLiMs rich in His, Lys and Arg residues, (iii) HO-SLiMs comprising residues that promote order and are hydrophobic, and (iv) zipper-SLiMs that readily form the cross-β-sheet structure of amyloid fibrils. In other IDPs, other types of SLiMs have been recognized including those composed of residues with π electrons such as Pro and Tyr that can form stacks of parallel side chains or bind to hydrophobic ligands with π electrons such as polyphenolic compounds, through π-bonding interactions (Dinkel et al., 2016). The list of candidate SLiMs in caseins is most probably incomplete.

Caseins are members of a paralogous group of secreted, calcium- or calcium phosphate (CaP)-binding, phosphorylated IDPs, known as SCPPs. Highly phosphorylated IDPs can influence every aspect of the phase separation of calcium salts from supersaturated solution including the non-classical mechanism of CaP precipitation near or above neutral pH in which a precursor amorphous phase is formed. The phosphoprotein may stabilize the amorphous phase, sequester it in a core-shell structure or modify how, and at what rate, the amorphous CaP matures into one or more stable crystalline phases. Further details can be found in (George and Veis, 2008, Gebauer et al., 2018, Lenton et al., 2020, Skibsted, 2023). Proteins that limit or prevent pathological calcification have been called mineral chaperones (Jahnen-Dechent et al., 2020, Rudloff et al., 2022). The more highly phosphorylated cow caseins (β, α_{S1} and α_{S2}) can certainly be considered mineral chaperones because they limit or prevent pathological CaP deposits in the mammary gland.

This review describes the stability of raw and processed milk and milk protein products using current concepts of protein stability. Use is made of the multivalent-binding model of casein micelle structure in raw milk at its natural pH (Bijl et al., 2019, Lenton et al., 2020, Holt, 2021, Holt and Carver, 2022, Carver and Holt, 2024, Raynes et al., 2024). This model describes the casein micelle as a complex of casein IDPs with a high degree of dynamic disorder, together with CaP nanoclusters. They spontaneously form a polydisperse distribution of non-stoichiometric, fuzzy, complexes called casein micelles. The casein micelle can also be regarded as a dispersed liquid-like phase (Horvath et al., 2022). The dispersion of casein micelles is very long-lived because of a high activation energy, probably steric or entropic in origin, preventing the fusion of the micelles or liquid-like droplets into a more stable condensed phase although the barrier is not so large as to prevent fusion occurring during heat treatments or upon prolonged storage. A key concept in the multivalent-binding model is that individual caseins are either bound directly to the CaP nanoclusters (Cas_b) or they are free (Cas_f). Free caseins are more soluble than bound caseins and hence are critically important for the average solubility, and hence stability, of the casein micelle. Free caseins can exchange more rapidly between the micelle and serum than bound caseins, and hence can act as molecular chaperones in preventing undesirable protein aggregation. The more highly phosphorylated caseins can also act as mineral chaperones to prevent the formation of a macroscopic phase of CaP.

The multivalent-binding model is supported by a wide range of experimental findings on raw milk at ambient temperature. It is the only model that provides a reasonably precise and quantitative description of the partition of milk salts between the micelles and milk serum (Bijl et al., 2019) and of caseins between free and bound forms, as measured by urea dissociation (Aoki et al., 1986, Aoki et al., 1987). It predicts a polydisperse, unimodal distribution of micelle sizes, in reasonable agreement with the average size and size distribution determined by experiment (Saveyn et al., 2010, Thill et al., 2020, Holt, 2021, Holt and Carver, 2022) but without the extended tail of larger micelles. Much experimental work has confirmed that caseins can act as molecular chaperones to inhibit the unfolding and aggregation of globular proteins and the formation of amyloid fibrils (Bhattacharyya and Das, 1999, Farrell et al., 2003, Morgan et al., 2005, Thorn et al., 2005, Léonil et al., 2008, Thorn et al., 2008, Treweek et al., 2011, Bahraminejad et al., 2022). These observations are difficult to explain using hydrophobic colloid models.

Recently, critical experiments have discriminated between the multivalent-binding and hydrophobic colloid models of the casein micelle. Small-angle x-ray scattering and small-angle neutron scattering with contrast variation experiments were carried out on biomimetic casein micelles made with native κ- and recombinant, deuterated and phosphorylated β-casein (Raynes et al., 2024). The dependence of the radius of gyration on the proportion of κ-casein and its variation with scattering contrast showed that the κ-casein is present in both the coat and core of the micelles, as predicted by the multivalent-binding model. The findings are contrary to hydrophobic colloid models (Linderstrøm Lang, 1929, Payens, 1966, Waugh and Talbot, 1971, Slattery and Evard, 1973, Schmidt, 1982, Walstra, 1990, Horne, 1998) in which κ-casein exclusively forms the micelle coat and is absent, or nearly so, from the core. In hydrophobic colloid models, the coat of κ-casein is hypothesized to stabilize the core of the supposedly hydrophobic caseins against aggregation by a steric

mechanism (Walstra, 1990, Holt and Horne, 1996) and proteolytic removal by chymosin of the κ-casein C-terminal macropeptide destabilizes the micelles by exposing the core caseins. However, horse casein micelles, for example, contain very little κ-casein (Ochirkhuyag et al., 2000, Uniacke-Lowe et al., 2010). It has been suggested that in this species, the function of κ-casein may be supplemented by Ca-sensitive caseins with a low level of phosphorylation (Ochirkhuyag et al., 2000). According to the multivalent-binding model, any of the free caseins can increase micelle stability so κ-casein is a desirable, but not essential, component of stable micelles. Moreover, stable artificial casein micelles can be made from Ca-sensitive cow caseins, singly or in mixtures, without any κ-casein (Raynes et al., 2023).

SOLUBILITY AND STABILITY OF CASEINS AND CASEIN MICELLES

The solubility of proteins has evolved to match their biological functions. Hyperexpression of caseins in mammary secretory cells and high, mM, concentrations of caseins in the milk of fast-growing and marine mammals (Jenness and Sloan, 1970, Oftedal, 2020) requires caseins to be highly soluble. Protein solubility at neutral pH can be correlated empirically with amino acid residue composition and sequence, particularly for IDPs (Hebditch et al., 2017). A high proportion of charged or neutral, polar residues and a low proportion of aromatic residues favor high solubility. The web-based predictor Protein-sol (https://protein-sol.manchester.ac.uk/) uses an optimized multivariate method based on 10 amino acid composition and sequence properties to predict the solubility of *E. coli* proteins at neutral pH. Results were used to create a solubility scale parameter, X, ($0 \leq X \leq 1$) with a population average of 0.45 for the *E. coli* proteome. Calculated values of X for the 4 unphosphorylated mature cow caseins are similar and well above this average at 0.734 (α_{S1}), 0.731 (α_{S2}), 0.774 (β) and 0.655 (κ). Plasmin and chymosin fragments of caseins are likewise predicted to be highly soluble: 0.644 (κ-casein 1–105), 0.607 (β-casein 29–209), 0.501 (β-casein 106–209), 0.820 (β-casein 1–28) and 1.00 (κ-casein 106–199, the macropeptide). Thus, caseins, even before phosphorylation, have a high solubility score. Proteolysis of caseins by chymosin or plasmin slightly decreases their solubility score, so other factors are required to explain why caseins aggregate to form casein micelles, amyloid fibrils, rennet clots and acid gels. To help understand these phenomena, we briefly summarize the current understanding of protein solubility as affected by charge and co-solutes such as salts.

QUASI TWO-PHASE DIAGRAM OF PROTEIN SOLUTIONS

Phase diagrams show the dependence of protein solubility on environmental variables. The example phase diagram in Figure 1 is of a single protein which can lose its solubility because of salting-out, pH adjustment or a change of the intrinsic variables of temperature and pressure. The binodal is the boundary of a 2-phase region in which an equilibrium is formed between a condensed, concentrated protein phase, and a more dilute protein solution. Within the binodal region many IDPs, including caseins form a metastable dispersion in a more dilute continuous phase. The spinodal is the boundary marking the limit of metastability for example the maximum supersaturation of a salt or protein solution or spontaneous transformation of a metastable gel into a more condensed state or an IDP into a more stable amyloid structure (Michaels et al., 2023). Within the spinodal, the condensed protein phase is in equilibrium with a more dilute saturated protein solution.

The position and shape of the binodal can be calculated from how the free energy change as a result of mixing of the protein with the solvent, $\Delta\mu_{mix}$, depends on the protein concentration using the method of common tangents (Rubinstein and Colby, 2003). A relatively simple theory of mixing can be used to illustrate the principles governing phase separation. For a protein of N_{res} residues occupying a volume fraction φ in a lattice of co-ordination number z, the Flory-Huggins theory (Huggins, 1942, Flory, 2004) gives

$$\Delta\mu_{mix}/RT = \left[\frac{\varphi}{N_{res}}\ln\varphi + (1-\varphi)\ln(1-\varphi) + \chi\varphi(1-\varphi)\right], \quad (1)$$

where R is the gas constant, and the interaction parameter χ is

$$\chi = z(2u_{PS} - u_{PP} - u_{SS})/2\mathrm{RT}, \quad (2)$$

where u_{PS}, u_{PP}, and u_{SS} are mean field energies of interaction of the protein with the solvent, the protein with itself and the solvent with itself, respectively. The interaction forces include dipolar, electrostatic, cation-π- and aromatic-π-bonding, the hydrophobic effect and the Van der Waals dispersion force (Das et al., 2020). In a good solvent, interactions of the protein with the solvent are favored: u_{PS} is large and negative and a homogeneous single phase is formed at all protein concentrations. In an indifferent, or θ, solvent, the protein-protein and solvent-solvent interactions just counterbalance the protein-solvent interaction and $\chi \approx$

0, but entropy favors a well-mixed single-phase solution. In a poor solvent, $\chi > 0$, protein-protein interactions are favored over protein-solvent interactions and phase separation occurs above the solubility limit of the protein. For milk, ethanol at low temperatures is a poor solvent for casein micelles, whey proteins and calcium salts but at 40 −50°C, a mixture of about 35% ethanol in milk becomes a better solvent for casein micelles (O′Connell et al., 2001a, O′Connell et al., 2001b, Lewis et al., 2022).

The theory of phase separation of IDPs and low-complexity proteins has advanced considerably in recent years as the basis for understanding the formation of protein liquid-like droplets or condensates and intracellular membrane-less organelles (Brangwynne et al., 2015, Banani et al., 2017, Alberti et al., 2019, Lyon et al., 2021). The interested reader is referred to these citations for more information. The casein micelle can be regarded as a comparable, but extracellular, condensed phase formed by casein IDPs, some of which are bound to the CaP nanoclusters and the remainder are free (Horwath et al., 2022). In a multi-component protein mixture such as milk, the phase diagram is likely to be more complex than Figure 1, not least because globular whey proteins partially unfold, aggregate and may become insoluble under milder conditions than the more soluble caseins. In milk at ambient temperature, the whey proteins can be considered part of the solvent with the caseins comprising 2 quasi-components, namely the caseins bound to the CaP nanoclusters and the free caseins (Holt, 2021, Holt and Carver, 2022).

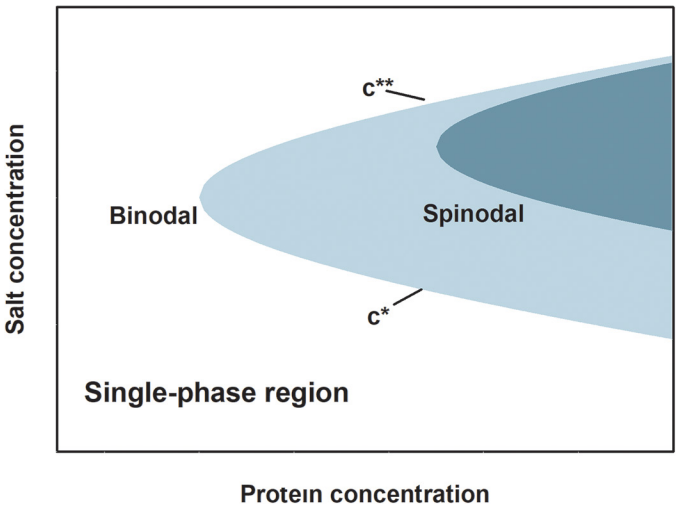

Figure 1. An example of a 2-component phase diagram, representing the phase behavior of a closed system comprising a protein and an aqueous solvent, projected onto the dimensions of protein concentration and salt concentration. The binodal marks the boundary between one phase and 2 coexistent phases. At lower protein concentrations, the single-phase region may be a solution of the protein in the solvent but at high protein concentrations it may be a viscous single phase comparable to a polymer melt (Rubinstein and Colby, 2003). Within the binodal, a denser condensed protein phase co-exists with a more dilute phase. Network-forming polymers like IDPs tend to produce metastable dispersed or arrested condensed phases or gels rather than a condensed macroscopic phase. At lower protein concentrations the denser condensed phase may be dispersed in a more dilute phase but at higher protein concentrations the dispersion may be inverted. Within the binodal region there may be a sub-region marked by the spinodal which is the stability limit of the metastable dispersed phases. At the salting-out concentration C* a metastable condensed phase is formed but at the higher salt concentration C**, salting-in occurs in the phenomenon called re-entrant condensation.

CASEINS ARE AMYLOID-FORMING PROTEINS

Amyloid fibril formation under destabilizing conditions is common among both globular proteins and IDPs because the amyloid fibril structure is of lower overall free energy than either the folded or unfolded conformations (Hartl et al., 2011, Ke et al., 2020). Short sub-sequences of 6 residues, called steric zippers or zipper-SLiMs, can be threaded into the cross-β-sheet crystal structures formed by amyloid fibrils with a favorable Rosetta crystal energy. Steric zippers are found in most proteins (Goldschmidt et al., 2010), and are abundant in cow caseins (Holt et al., 2019), so they have at least the potential to form fibrils.

Amyloid fibrils and amorphous aggregates are formed from off-folding protein pathways originating either from a folded native (globular) state or an unfolded (intrinsically disordered) state (Figure 2) (Hall et al., 2015, Hall et al., 2016). In both pathways, the conversion from the native to the aggregated state is via partially unfolded intermediate(s) of differing structural characteristics depending on the type of aggregate formed. After a lag phase in which the intermediates form, they associate to form a nucleus via a nucleation-dependent mechanism. The nucleus sequesters native proteins to form, in the case of fibril formation, protofibrils, and then a rapid, exponential growth of fibrils occurs until an equilibrium or plateau phase is reached (Figure 2). According to various theoretical treatments or molecular dynamic simulations of amyloid fibril formation (Lee, 2009, Zamparo et al., 2010, Schmit et al., 2011, Schreck and Yuan, 2011, Steckmann et al., 2012, Schreck and Yuan, 2013a, b, Zhang and Schmit, 2017, Zhang, 2018), an amyloidogenic protein can form either an amorphous aggregate or amyloid fibrils of various morphologies, or a mixture of the 2. The outcome, at equilibrium, depends on the critical monomer concentrations for the formation of each type of aggregate and the energies of formation of each intermediate. The optimum rate of fibril formation is from partially- rather than fully-unfolded intermediates because sequences not involved in nucleation (particularly those that are flexible and disordered) can inhibit the

growth rate (Hamada and Dobson, 2002, Hamada et al., 2009, Chiti F, 2017, Gowda et al., 2021). Amyloid fibrils are a stable outcome because of the formation of backbone hydrogen bonds and various types of side chain interaction. If the concentration of fibrils formed is high enough, they may further aggregate into a liquid crystalline state (Kuriabova et al., 2010) or entangle to form a network or gel. When the kinetics of this system are studied (Zhang, 2018, Phan and Schmit, 2022), it is commonly found that amorphous aggregation is more rapid than fibril formation due to the slow nucleation step in the formation of protofibrils. However, over time, a re-equilibration occurs to diminish the initially high concentration of amorphous aggregates and increase the proportion of fibrils formed either within the amorphous aggregates or by a slower process of dissociation from the amorphous aggregate followed by fibril formation (Lee, 2009, Schmit et al., 2011). Fibril growth within the amorphous aggregate is more rapid because of the higher concentration of amyloidogenic sequences. The protofibrils may act as sites of heterogeneous nucleation to speed up fibril formation autocatalytically (Xu et al., 2014).

Molecular chaperones, such as small heat-shock proteins (sHsps), inhibit and reduce the growth of amyloid fibrils in a variety ways by (i) interacting with early-stage, partially unfolded intermediates, (ii) binding to the fibrils (including to their ends) to block their further growth, and (iii) redirecting the intermediates to the amorphous aggregation pathway (Carver et al., 2002, Rekas et al., 2004, Waudby et al., 2010, Baldwin et al., 2011, Williams et al., 2021). Another limitation of fibril growth is the formation of incompatible inter- or intra-molecular non-native disulfide bridges and competition at the growth sites by the binding of incompatible sequences.

Amyloid fibrils can also result from the proteolysis of larger proteins (Ke et al., 2020, Acquasaliente et al., 2022). In many cases, the resultant peptides contain steric zipper sequences. In vivo, proteins which give rise to amyloidogenic peptides include the amyloid precursor protein (to produce amyloid β peptides, associated with Alzheimer's disease), α-synuclein (associated with Parkinson's disease) and transthyretin (associated with amyloidosis). In relation to milk proteins, (Ye et al., 2018) found that hydrolyzed peptides resulting from prolonged acid treatment of β-lactoglobulin (β-Lg) at elevated temperature formed amyloid fibrils. Moreover, (Murakami et al., 2023) observed that N-terminal truncation of α_{S1}-casein, coupled with its overexpression in a mammary tumor, led to it forming amyloid fibrils. By contrast, intact α_{S1}-casein does not readily form fibrils. Thus, the unstructured, flanking N-terminal region of α_{S1}-casein 'protects' the central, amyloidogenic region from forming fibrils, as occurs with other peptides and proteins (Carver et al., 2017). Because the 4 cow caseins contain 5 or more steric zipper sequences (Carver and Holt, 2019, Holt et al., 2019), it is highly likely that proteolytic casein fragments will form amyloid fibrils. For example, after digesting casein micelles with trypsin, a high molecular mass fraction was isolated by centrifugation with the expectation that the pelleted peptides would be the most highly phosphorylated peptides bound to CaP nanoclusters (Gagnaire et al., 1996). Surprisingly, a considerable number of the pelleted peptides identified from their mass were unphosphorylated. In Table S5 of the supplement to (Holt et al., 2013), a comparison was made between the steric zipper predictions and the pelleted peptide sequences. Of the 46 identified peptides from the pellet, 24 were unphosphorylated and 20 of these contained steric zipper sequences. A reasonable conclusion is that the pelleted material contained a significant fraction of amyloid fibrils.

Amyloid formation by cow caseins has been extensively investigated. Under physiological conditions, in the absence of other caseins, cow κ-casein readily forms amyloid fibrils, with α_{S2}-casein also prone to do so (Farrell et al., 2003, Thorn et al., 2005, Léonil et al., 2008, Thorn et al., 2008). By contrast, the predominant cow caseins, α_{S1} and β, require harsher conditions of elevated temperature and longer times to form fibrils at neutral pH (Bahraminejad et al., 2022). Many other studies have deepened our understanding of the amyloidogenicity of caseins, confirming its importance in casein chemistry and dairy technology (Léonil et al., 2008, Stroylova et al., 2012, Chen et al., 2015, Lee et al., 2018, Lee et al., 2019, Wang et al., 2020a, Wang et

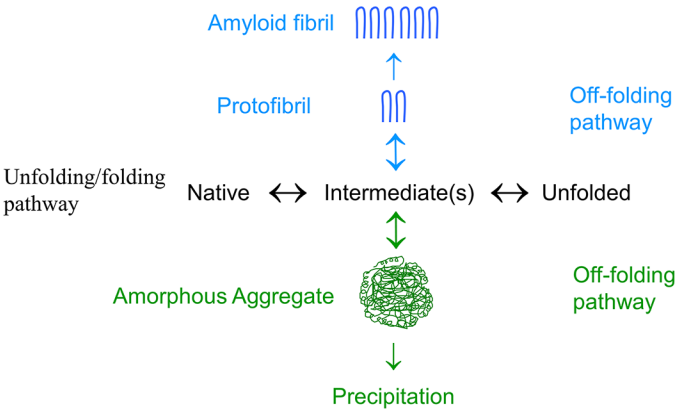

Figure 2. Amorphous and amyloid fibril off-folding protein aggregation pathways. Both types of aggregates form from intermediates that deviate from the unfolding/ folding pathway. Detailed information on the structure of intermediates, and their differences, which lead to forming either amorphous or fibrillar aggregates, is not known.

al., 2020b, Guarrasi et al., 2021, Zanyatkin et al., 2021, Wang et al., 2022). Further details can be found in other reviews (Raynes et al., 2014, Redwan et al., 2015, Thorn et al., 2015, Lambrecht et al., 2019, Zhou et al., 2019, Carver and Holt, 2024).

OCCURRENCE OF STRAND-LIKE STRUCTURES IN MILK AND MILK PRODUCTS

There is increasing evidence of amyloid structures in milk and milk products, as would be expected after stresses such as high temperature, pressure and shear, or prolonged storage with or without proteolysis. As well as casein amyloid fibrils, fibrils are formed near neutral pH by partial thermal or urea denaturation of the preponderant cow whey protein, β-Lg (Gosal et al., 2004), or by heating the sequence from β-strand-A of its calyx-shaped binding pocket (Hamada et al., 2009). Fibrils are also formed from hydrolyzed peptides when β-Lg is heated at around 80°C at pH 1–3 (Gosal et al., 2004, Loveday et al., 2011). At neutral pH, reduced α-lactalbumin, another common whey protein, also forms amyloid fibrils (Kulig and Ecroyd, 2012) but in heated whey protein mixtures at pH 2, only β-Lg is reported to form fibrils (Bolder et al., 2006).

Imaging methods have identified protein structures in milk protein systems with a single extended dimension, variously called strands, strings, filaments, tendrils, chains, rods, fibers, fibrils or protofibrils (Loveday et al., 2017, Cao and Mezzenga, 2019, Lambrecht et al., 2019). Such structures have been reported in milk (Lencki, 2007), various types of heat-treated milk (reviewed by (McMahon, 1996, Datta and Deeth, 2001, Nieuwenhuijse and van Boekel, 2003, Deeth and Lewis, 2016)), concentrated milk (Schmidt and Bucheim, 1968, Rathod et al., 2023), milk powder (Wawer et al., 2023), cottage cheese (Malik et al., 2022), processed cheese (Tamime et al., 1990, Heertje, 2014, Vollmer et al., 2021a, Vollmer et al., 2021b), sodium caseinate (Farrer and Lips, 1999), yogurt (Davies et al., 1978) and whey proteins or hydrolysates (Bolder et al., 2006, Loveday et al., 2012, Hu et al., 2019, Khalesi et al., 2021). In UHT milk, the fibrils are sometimes attached to the casein micelles and may cause bridging flocculation or gelation of the micelles (Andrews et al., 1977, Davies et al., 1978, Harwalkar, 1992, Deeth and Lewis, 2016). Similar fibrils projecting from and bridging casein micelles were observed in the fouling deposit on reverse osmosis membranes (Skudder et al., 1977). Separate fluorescent labeling of β-Lg and κ-casein with different fluorophores or immunolabels shows that both are present in the connecting strands (Dubert-Ferrandon et al., 2006, Malmgren et al., 2017). In a simulation of UHT treatment, these purified proteins were heated together in solution at 94°C for one hour. Amyloid cofibril formation between these 2 structurally unrelated proteins (an unusual phenomenon) was demonstrated by single-molecule fluorescence techniques (Raynes et al., 2017). It is now generally accepted that amyloid fibril formation is an inevitable outcome of some milk processing operations. Indeed, fibrillar aggregates of milk proteins have been purposefully generated in pursuit of novel milk protein food functionalities (Nicolai and Durand, 2013).

CASEINS ARE HOLDASE-TYPE MOLECULAR CHAPERONES

There are 2 general categories of molecular chaperone proteins: 'foldases' and 'holdases' (Hoffmann et al., 2004). Caseins are promiscuous holdase chaperones because they stabilize and often bind to a wide range of client proteins to prevent their aggregation (Morgan et al., 2005, Yong and Foegeding, 2010, Treweek et al., 2011, Akbari et al., 2018). Holdases have no ATPase activity and therefore, unlike foldases, have no ability to fold or refold client proteins, processes which require ATP hydrolysis.

The chaperone action of caseins is analogous to that of intracellular sHsps and extracellular clusterin. It involves multivalent interactions of caseins with client proteins, as also occurs between caseins in the casein micelle (Horvath et al., 2022). There is no preferred or defined chaperone binding site(s) within caseins. The multivalent interactions between various sites on the caseins and their client proteins are the mechanistic basis of casein chaperone action. The dynamic, disordered conformation of caseins facilitates their interactions with a wide range of client proteins during chaperone action.

The efficiency of holdase chaperone action, including that of caseins, is greater for slowly aggregating client proteins, whether they are aggregating in an amorphous or fibrillar manner (Carver et al., 2002, Cox et al., 2016, Sanders et al., 2020). In the case of sHsps, the enhanced efficiency is related to the rate of exchange of subunits between the solvent and aggregate, since the dissociated form of the chaperone is the more chaperone-active species (Treweek et al., 2015, Hayashi and Carver, 2020). Due to the analogous chaperone action of caseins and sHsps (Carver et al., 2018), it is understandable therefore that free caseins in milk are better able to act as molecular chaperones than bound caseins.

An important role of all caseins in milk is to act as molecular chaperones to suppress casein amyloid formation and this may help to explain why casein in milk is always a mixture of 2 or more types of casein

(Holt and Carver, 2012). In general, holdase chaperone activity is likely to be effective if the molar ratio of holdase to client is greater than unity. In cow milk, the 2 caseins with a low propensity to form amyloid fibrils (α_{S1} and β) are about 4 times more abundant than the more amyloidogenic κ- and α_{S2}-caseins (Miranda et al., 2020). In vitro this ratio was sufficiently high to reduce substantially at neutral pH the rate of formation of amyloid fibrils by κ-casein (Thorn et al., 2005) or α_{S2}-casein (Thorn et al., 2008) in the assay conditions used. In milk processing, free caseins act as the main molecular chaperones. In the standard milk of (Bijl et al., 2019) at ambient temperature and pH 6.7, $[Cas_f]/[\kappa] = 2.92$, $[\kappa]/[\beta\text{-Lg}] = 0.89$, $[Cas_f]/[\beta\text{-Lg}] = 2.60$ and $[Cas_f]/[\text{Whey}] = 1.58$. These ratios are high enough for the free caseins to slow amyloid fibril growth by κ-casein in milk but may not completely suppress it, especially in the longer term (Thorn et al., 2005). Similar arguments apply to the inhibition of β-Lg fibril formation by the free caseins.

PROTEIN-SALT INTERACTIONS

Salts and pH can have specific and general effects in determining the solubility of proteins, over and above the effect of ionic strength on the shielding or screening of electrostatic interactions. Inclusion of all these effects provides a more complete explanation for protein stability than that from electrostatic repulsion alone. In general, proteins have a low charge density at physiological pH and ionic strength so that the surface potential, ψ_s, is small ($e\psi_s/\text{RT} < 1$, where e is the electron charge). As a result, the mean field energy of electrostatic repulsion is lower than thermal energy. It is usual to separate the effects of ions, including the hydronium ion, H^+, that bind strongly and specifically to particular binding sites from the weaker salting-in and salting-out effects on all residues. Salts can affect protein solubility through each of the 3 terms in equation (2). Because of their low hydrophobicity, IDPs are salted-in by chaotropes but are less prone than most other proteins to salting-out. More subtle salt effects, however, are possible, such as perturbing the ensemble of conformations or altering the propensity to fold into a stable conformation (Wicky et al., 2017, Camacho-Zarco et al., 2022).

STRONG AND SPECIFIC ION BINDING TO CASEINS AND CHARGE NEUTRALIZATION

Proteins tend to have their minimum solubility at or around their point of zero net charge (pzc). Charged residues contribute strongly to u_{PS} in equation (2) and they are fewest at or near the pzc, taking into account specific ion binding. Likewise, the specific binding of multivalent cations to caseins (Dalgleish and Parker, 1980, Parker and Dalgleish, 1981) and other anionic proteins reduces their solubility so that near their pzc, phase separation may occur at a critical salt concentration, c*. At a higher cation concentration, c**, charge reversal or, more generally, salting-in may take place where a condensed phase redissolves in a phenomenon called re-entrant condensation (Figure 1) (Zhang et al., 2008, Roosen-Runge et al., 2013, Matsarskaia et al., 2020). Re-entrant condensation has also been observed with polyvalent anions. For example, it occurs with lysozyme at pH 9, due to the binding of pyro- or polyphosphates at sites containing Lys and Arg residues (Bye and Curtis, 2019) and with the basic salivary IDP, histatin (Lenton et al., 2021), due to binding citrate or polyphosphates at sites containing Arg residues. According to the re-entrant liquid condensation model (Matsarskaia et al., 2020), the multivalent ions induce phase separation by reducing net charge and by non-covalent cross-linking of protein molecules, whereas re-entrant condensation by polyvalent ions occurs due to charge reversal.

Figure 3 shows the effect on the solubility of cow mineral chaperones produced by low mM concentrations of $CaCl_2$. At c*, where phase separation begins, the average charge on each of the caseins in Figure 3 is negative but the bound calcium ions could have a cross-linking action (Zhang et al., 2008) as a contribution to multivalent protein-protein interactions.

The phenomenology of re-entrant condensation has generated new insights into metastable and arrested phases, gelation and glass transitions induced by salts or temperature (Zhang et al., 2008, Matsarskaia et al., 2020). Some of these insights are directly applicable to the behavior of milk proteins during storage and processing operations.

WEAK AND NON-SPECIFIC ION EFFECTS

The relative efficacy of different salts at salting-in or salting-out of proteins is usually expressed by a Hofmeister series (Figure 4).

However, there are many known exceptions to the orders shown in Figure 4, especially at low ionic strength where the specific binding of ions to proteins is strongest and may even generate a reverse Hofmeister series (Okur et al., 2017, Matsarskaia et al., 2020). Generally, Hofmeister salts affect the hydrogen bonding patterns of water molecules. They underlie many complex biological processes such as protein folding, unfolding, association, amyloid fibril formation and phase separation, but their physical basis is not fully understood (Baldwin, 1996, Collins, 2004, Shimizu et al., 2006,

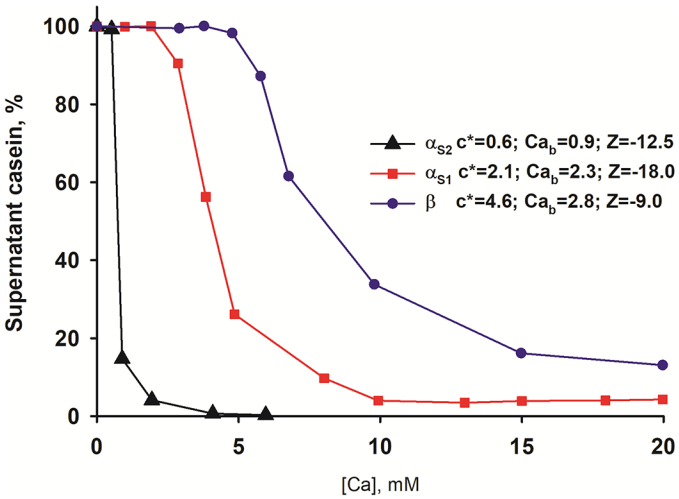

Figure 3. phase separation of the cow Ca-sensitive caseins by $CaCl_2$ near neutral pH. Redrawn from the original findings of (Aoki et al., 1985) for α_{S2} and α_{S1} at 25°C and (Yoshikawa et al., 1981) for β at 35°C. The lower critical calcium concentration, c*, mM, mole of bound Ca per mole of casein, Ca_b, and net charge, Z, of α_{S2}, α_{S1} and β at pH 7.0 are listed. There is no c* for κ nor for β at 1°C (Farrell et al., 1988). Condensed phases begin to form despite a net negative charge, due to multivalent interactions, including calcium cross-linking.

Okur et al., 2017). Residues can be divided into a polar part, forming the backbone of peptide bonds, and a side chain. Systematic studies on the effects of salts on model compounds have shown that the backbone and polar side chains are subject to a weak salting-in effect, proportional to ionic strength. Salting-out effects are strongest for aromatic side chains. For aliphatic ones, the salting-out effect of salts increases with the number of carbon atoms in the side chain but is not affected by branching (Nandi and Robinson, 1972b, a, Baldwin, 1996).

A systematic study of the effect of salts on the phase behavior of caseins has not been undertaken. The effect of low concentrations of calcium salts to reduce the solubility caseins is well known (Figure 3) and is attributed mainly to specific binding at sites formed mainly from phosphorylated residues, causing a charge reduction and allowing calcium bridging, gelation, and condensation. However, the calcium ion is a chaotrope (Figure 4) and at higher concentrations of $CaCl_2$, α_{S1}, and most likely all other caseins, are salted-in (Figure 5) (Farrell et al., 1988). The salting-in effect of the calcium salt on α_{S1}-casein is augmented by increasing the concentration of KCl, (Figure 5) as shown by the decreasing proportions of condensed phase pelleted by the centrifugation procedure as the neutral salt concentration increases.

The Kirkwood-Buff description of protein solutions (Kirkwood and Buff, 1951) has become the cornerstone of many current attempts to understand protein solubility and stability (Pierce et al., 2008). It describes a fluctuating cloud of co-solutes and water molecules around each protein particle. There is no hydration layer comprising water molecules alone. Instead salt ions, particularly large anions, penetrate the inner hydration layer and disrupt water structure (Okur et al., 2017). Shimizu and coworkers have proposed an explanation for Hofmeister effects based on this theory (Shimizu et al., 2006, Shimizu et al., 2017). According to this model, aggregation of proteins is driven by the exclusion of ions, more than the exclusion of water molecules, from the protein cloud. The model was applied to literature data on the association behavior of β-casein and to the effects of salts and pressure on the rate of aggregation of renneted casein micelles (Harton and Shimizu, 2019). For practical reasons, a simple isodesmic model was used to describe the self-association of β-casein and the von Smoluchowski model was used for aggregation rates to make quantitative comparisons of the effects of water structure and ion association in the fluctuating cloud. Harton and Shimizu (2019) noted that the rate of aggregation of renneted casein micelles was strongly increased by adding 4mM $CaCl_2$ but only modestly by an applied pressure of 100 MPa. They also argued that since the volume change on aggregation was too small to measure, changes of water structure and protein solvation played a negligible part in casein aggregation. Moreover, the initial rate of aggregation of renneted casein micelles was inhibited by NaCl but enhanced by $CaCl_2$. They also noted that there was a Hofmeister effect, in addition to an ionic strength effect, in the self-association of β-casein, again pointing to the importance of casein-salt interactions in driving self-association. The effect of water structure changes on casein association and aggregation was concluded to be negligible compared with the effect of salts. The paper of Harton and Shimizu attracted a critical comment because it downplays the importance of the hydrophobic effect in casein association (Horne,

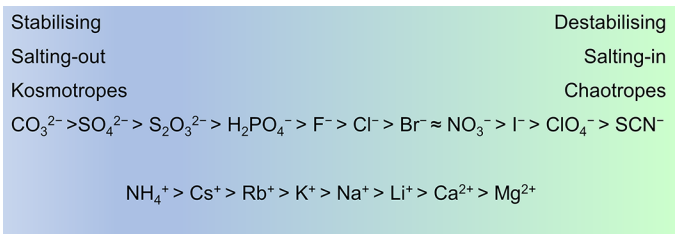

Figure 4. Hofmeister series. Kosmotropes are water structure inducers with salting-out effects that promote folding and aggregation, whereas chaotropes are water structure breakers with salting-in effects. Typically, Cl^- among anions and Na^+ among cations are at the dividing line separating salting-in from salting-out behavior.

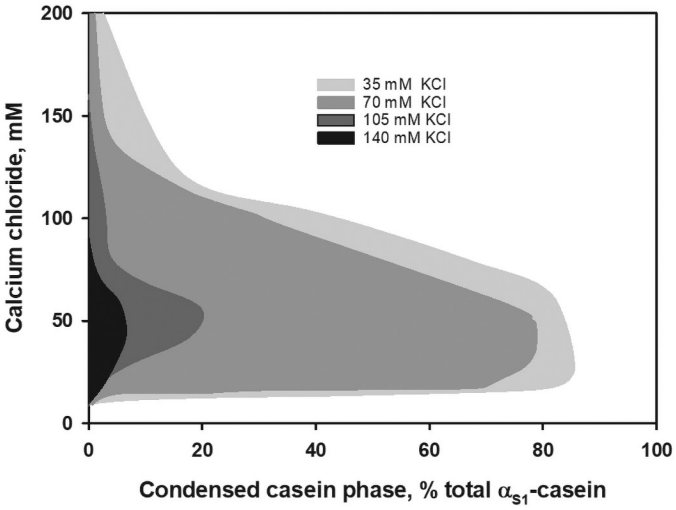

Figure 5. phase separation of α_{S1}-casein A (10 gL^{-1} at 1°C, pH 7.0) to produce co-existing condensed and more dilute phases in variable proportions, determined by a differential centrifugation procedure. Data are redrawn from (Farrell et al., 1988). There was a salting-out effect of $CaCl_2$ at less than 50 mM with a lower critical concentration of c* ~5–10 mM. Salting-in became the dominant effect of $CaCl_2$ at higher concentrations. Addition of KCl always had a salting-in effect. The data do not show a clear upper critical concentration, c**, at least by the centrifugation procedure used to separate the dilute and condensed phases.

2020) but these comments were answered (Harton and Shimizu, 2020). The description of casein aggregation by Harton and Shimizu is consistent with current views on how salts, in general, affect protein solubility (Okur et al., 2017).

MODELING MILK STABILITY USING THE MULTIVALENT-BINDING MODEL

It follows from their biological functions that casein micelles need to be highly stable at the pH and salt concentrations of milk but they must lose their solubility at lower pH and form a gel or more condensed phase. The multivalent-binding model provides insights into how the intrinsically high stability of milk can be reduced by 5 different but overlapping mechanisms: (i) Casein micelles aggregate because they lose their intrinsic solubility, for example at acid pH where the average net charge is small or, at physiological pH, when the solvent quality is reduced by the addition of a non-solvent or by raising the temperature. (ii) Amyloid fibrillar aggregates are formed by amyloidogenic caseins such as κ-casein or partially unfolded whey proteins. (iii) Amorphous aggregates are formed by unfolding whey proteins at elevated temperature or pressure. (iv) Casein micelles aggregate because they are depleted in the more soluble free caseins. (v) CaP precipitates because the free casein mineral chaperones are unable to sequester the CaP at an early stage of precipitation.

The highly dynamic casein micelle can be compared with a dispersed liquid-like phase (Horvath et al., 2022). Fusion of the droplets in the dispersion can occur when the solvent quality is poor which, for proteins, commonly occurs close to the pzc, but fusion may be delayed if the micelles have a small net negative or positive charge, leading to gelation rather than a condensed phase. Thus, using the average net charge of the casein micelle as a measure of relative solubility, conditions for phase separation or gelation can be calculated by the multivalent-binding model. Moreover, the multivalent binding model can be used to model other instabilities by calculating the ratio of the total molar concentrations of protein and mineral chaperones and comparing them to the molar concentrations of protein or CaP that could undergo phase separation or form amyloid fibrils.

Bound caseins are a fraction, α, of the total mineral chaperones in the milk. Thus, a milk is stable with respect to CaP precipitation provided $\alpha \leq 1$.

Free caseins in cow milk are κ-casein, mole fraction w_o of Cas_t, together with a proportion, (1-α), of the casein mineral chaperones that are not directly bound to the CaP nanoclusters. Hence, using square brackets for concentration

$$\begin{aligned}[Cas_b] &= \alpha(1-w_o)[Cas_t] \\ [Cas_f] &= \left[1-\alpha\left(1-w_o\right)\right][Cas_t].\end{aligned} \tag{3}$$

A significant parameter in the control of amyloid fibril formation by κ-casein is the molar ratio of κ-casein to the total concentration of other free caseins that can act as molecular chaperones.

$$\frac{[\kappa\text{-CN}]}{[Cas_f]-[\kappa\text{-CN}]} = \frac{w_o}{(1-\alpha)(1-w_o)} \tag{4}$$

Inhibition of amyloid fibril formation is effective when the value of the ratio in equation (4) is small (Thorn et al., 2005, Thorn et al., 2008, Treweek et al., 2011, Bahraminejad et al., 2022).

Among the major whey proteins, all have been found to form amyloid fibrils under appropriate conditions but the most abundant, β-Lg, has been the focus of most attention and is the most likely to form amyloid structures in whey protein products. The significant ratio controlling fibril formation by β-Lg is

$$\frac{[\beta\text{-Lg}]}{[Cas_f]} = \frac{[\beta\text{-Lg}]}{\left[1-\alpha(1-w_o)\right][Cas_t]} \tag{5}$$

For effective suppression of β-Lg fibril formation in milk, the ratio in equation (5) should be small.

The whey proteins in milk may partially unfold and aggregate during processing operations such as high temperature, pressure or shear treatments. As an approximation, all globular protein concentrations in milk are summed to give the whey concentration, [Whey]. A significant parameter in the control of whey protein unfolding and aggregation is therefore the molar ratio of total whey protein to free caseins in the milk.

$$\frac{[\text{Whey}]}{[\text{Cas}_f]} = \frac{[\text{Whey}]}{[1-\alpha(1-w_o)][\text{Cas}_t]} \tag{6}$$

It is assumed in equation (6) that all partially unfolded whey proteins bind to the casein molecular chaperones, whether they have the potential to form disulfide-linked oligomers, amyloid fibrils or amorphous aggregates. Stability is favored by a low value of the ratio in equation (6) and disfavored by a high ratio.

STABILITY OF RAW MILK

Figure 6a shows that the great majority of samples from individual cows are stable with respect to the precipitation of CaP at their natural pH. However, as the pH is raised, the number of CaP nanocluster complexes increases and Cas_f decreases to a minimum value. The exact behavior depends on the salt and casein composition of the milk (Bijl et al., 2019). In the standard milk composition used by (Bijl et al., 2019), there is an excess concentration of mineral chaperones at high pH, whereas certain other individual milk samples have an upper critical pH, pH**, at which all the mineral chaperones are bound to the CaP nanoclusters. Above pH**, the prediction of the multivalent-binding model is that the CaP nanoclusters increase in number and size but become less stable (Lenton et al., 2020, Wang et al., 2020c), eventually forming a CaP mineral phase.

Figure 6b shows that the great majority of individual cow raw milk samples are stable with respect to the formation of κ-casein amyloid fibrils. However, the free concentrations of the cow milk mineral chaperones, α_{S1}, α_{S2} and β-casein, decrease as the value of α increases, for example by either concentration of the milk due to evaporation or reverse osmosis, increasing the milk pH, or through calcium or phosphate fortification of the milk.

STABILITY OF MILK AT ACID PH

As the pH of milk is reduced, the number of CaP-nanoclusters progressively decreases to maintain the condition of CaP saturation in the serum and the stable size distribution of the nanoclusters (Lenton et al., 2020). $[\text{Cas}_b]$ decreases until, close to pH 5.7, all the CaP nanoclusters have dissolved. The concentration of free calcium ions in the serum increases up to this point and this tends to increase calcium binding to the acidic residues of caseins, including the CaP-SLiMs to the released mineral chaperones. However, the anionic residues also become protonated as pH is reduced and hence provide fewer and weaker binding sites for divalent cations. The net effect of these various processes is to produce a maximum in the number of calcium ions bound to free caseins at around pH 5.7 and the maximum potential for cross-linking the casein micelles through calcium bridges (Figure 7). In the absence of divalent cations, the pzc of all the caseins is around pH 4.6 but because of charge neutralization by the bound calcium ions, the pzc is raised to about pH 5.5. At lower pH than the pzc, the free caseins carry an average net positive charge and above the pzc the average net charge is negative. The effect of pH on calcium ion binding to free caseins and their absolute net charge in the standard cow milk (Bijl et al., 2019) are shown in Figure 7. It appears from Figure 7 that the optimum pH for forming a condensed casein phase from a standard milk composition is around pH 5.3 – 5.7 where the net charge is small. However, the cross-linking action of the bound calcium ions may help to form and stabilize a metastable gel rather than a condensed casein phase. Likewise, removal of the κ-casein macropeptide by chymosin decreases the average solubility of caseins and hence increases the tendency of caseins to form a condensed phase or stronger gel (Lewis, 2022).

The effect of adding 0.1 M NaCl to the standard raw milk is to decrease the pzc from 5.47 to 5.26 while moving the maximum of the calcium caseinate curve in the opposite direction from pH 5.68 to 5.93 and decreasing the concentration of the calcium caseinate from 6.57 mM to 5.25 mM. The net effect is therefore to make the conditions for gelation or condensation less optimal. Milks that fail to form a gel, or form a weak gel on renneting and acidification, can often be induced to gel or form a stronger gel by adding up to 10 mM calcium salts (Udabage et al., 2001). In the multivalent-binding model, addition of 10 mM $CaCl_2$ reverses the salting-in effect of added NaCl to restore the conditions needed for gelation or condensation. Thus, the multivalent-binding model of acid gelation precisely reproduces the effects of NaCl and $CaCl_2$ additions observed experimentally (Daviau et al., 2000, Karlsson et al., 2007, Sandra et al., 2012, Deeth and Lewis, 2015, Zhao and Corredig, 2015).

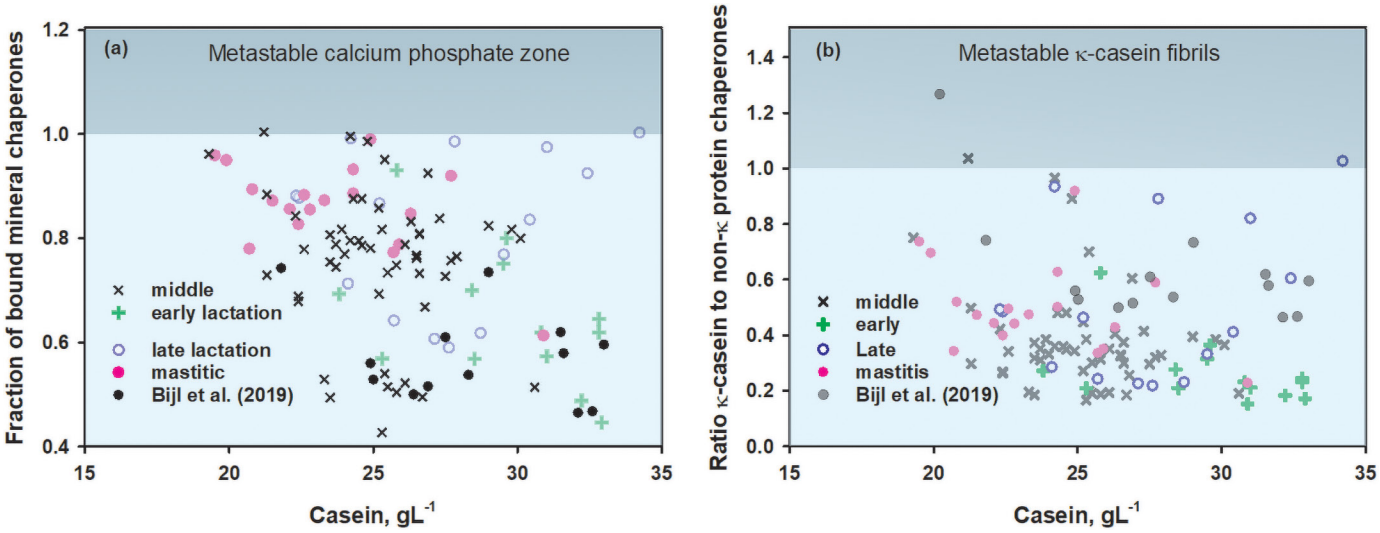

Figure 6. Significant compositional parameters affecting the stability of cow milk at its natural pH according to the multivalent-binding model (Bijl et al., 2019, Lenton et al., 2020, Holt, 2021, Holt and Carver, 2022). (a) Milk is stable if there is an excess of casein mineral chaperones, as is usually the case. (b) Stability with respect to amyloid fibril formation by κ-casein. Milk is stable if there is an excess of casein molecular chaperones to control casein amyloid fibril formation, as is usually the case. Total casein and salt composition data are from the milk of healthy individual cows in early, middle, or late lactation and from individual cows with mastitis (White and Davies, 1958, Davies and Law, 1977, Bijl et al., 2019).

STABILITY OF MILK AFTER WHEY PROTEIN DESTABILIZATION

Giant casein micelles up to several μm diameter are occasionally found in raw milk, but are much more common in heat-treated milk (Davies et al., 1978, Le et al., 2008, Glantz et al., 2010) and perinatal milk and colostrum that have been stored for some time in the mammary gland (Brooker and Holt, 1979). Void spaces are present in some giant micelles, indicating they are formed by a post-secretory process of partial or complete fusion of smaller micelles. Likewise, the internal structure of micelles from processed milk, as determined by small-angle scattering, appears to be more heterogeneous with respect to void spaces (Shukla et al., 2009, Bouchoux et al., 2015, Ingham et al., 2016) than freshly prepared raw casein micelles (Marchin et al., 2007, de Kruif, 2014). Casein molecular chaperones dissociate from micelles on heating (Aoki and Imamura, 1975) and bind to partially unfolded whey proteins (Sawyer, 1969) to diminish their concentration in the micelles, a factor that may be responsible for the formation of some of the giant casein micelles in heat-treated milk. Micelles depleted in free caseins are therefore predicted to be less stable (Holt, 2021, Holt and Carver, 2022).

Figure 8 shows the molar ratio of total whey protein to free caseins in individual milks calculated from equation (6). The ratio is above unity in a significant fraction of the samples, particularly milks from cows in late lactation or suffering from mastitis. In these milks, whey proteins (serum albumin), sodium and chloride concentrations and pH tend to be higher than in the milk of healthy cows in mid-lactation because of leaky tight junctions between mammary secretory cells (Stelwagen and Singh, 2014). The implication is that in milks with a high ratio of total whey protein to casein

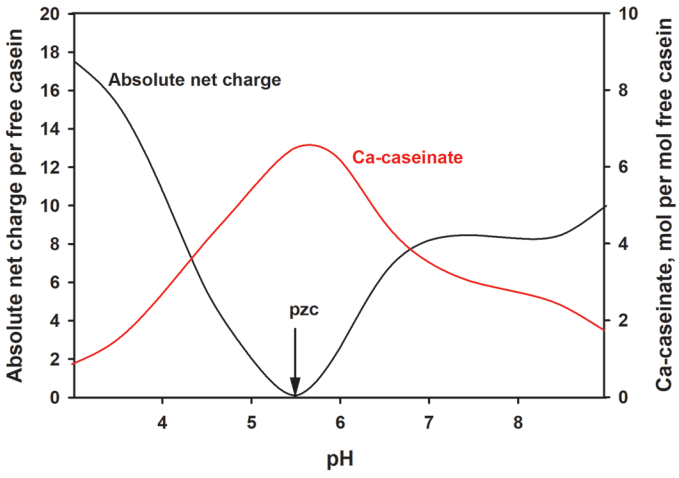

Figure 7. Effect of pH on the absolute value of the average net charge on free caseins and the average number of bound calcium ions per free casein in the standard cow milk of (Bijl et al., 2019). The protein charge is a minimum close to pH 5.5 and the maximum binding of calcium ions is close to pH 5.7. The optimum conditions for forming a condensed phase are therefore in the pH range 5.5 – 5.7. Conditions required for a metastable gel are in the flanking regions of this pH range where syneresis of the gel, once formed, can be reduced by adjustment of pH and free calcium ion concentration.

molecular chaperones, the casein micelles will be larger and less stable after whey protein unfolding.

During various other processing steps, the concentration of casein mineral and protein chaperones may be reduced. For example, raising the milk pH or concentrating it will increase the number of CaP nanocluster complexes with casein mineral chaperones.

CALCIUM PHOSPHATE PRECIPITATION

No discussion of casein micelle stability is complete without also considering the stability of the CaP nanocluster complexes. According to current concepts, the amorphous CaP core of the complexes is in an arrested state on the way to forming a more stable macroscopic phase of crystalline CaP such as apatite. It is stabilized by a shell of Ca-sensitive caseins via CaP-SLiMs so that the complex exists in a local free energy minimum, separated by a large transitional state energy from the more stable crystalline state. Further details of the theory are available (Holt, 2013, Lenton et al., 2020). Provided there is an excess of sequestering caseins, the size of the CaP nanocluster complexes is very nearly constant. Moreover, the complexes are so stable that they can survive heat treatments and dehydration. Notwithstanding this, when milk is brought into contact with hydroxyapatite powder, the number of CaP nanoclusters in the casein micelles decreases (Tercinier et al., 2014). The mechanism is likely to be a solution-mediated solid to solid transformation of the amorphous phase into a more stable crystalline phase (Eanes et al., 1965).

Figure 9 shows a rationalization of the current knowledge on the formation and stability of CaP-nanocluster complexes and casein micelles in terms of a phase diagram projected onto the axes of pH and casein concentration. The example chosen is a standard milk with average or typical concentrations of proteins and salts, including calcium and inorganic phosphate, P_i (Bijl et al., 2019). The standard milk becomes saturated with respect to the form of CaP in the micelle when the pH is at or above a critical pH, pH*. A binodal separates a single-phase protein solution below pH* from a 2-phase region comprising a condensed phase of CaP-nanocluster complexes in equilibrium with a dilute aqueous salt solution of some of the free caseins. The CaP nanocluster complexes spontaneously associate to form casein micelles but as pH increases within the binodal region, the association tendency decreases and the average size of the micelles decreases. At an upper critical pH boundary, labeled pH**, the complexes begin to lose stability and as the pH increases further and more CaP is precipitated from solution, there are too few casein mineral chaperones to form the most stable complexes. In reality, the upper boundary is not sharp because complexes of reduced stability form as pH increases. In the standard milk, the upper stability boundary is not reached because there is a high enough concentration of the casein mineral chaperones to sequester all the CaP that can be produced at high pH. This situation is not inevitable as there are individual milk samples with lower casein and/or higher calcium and P_i concentrations for which pH** is as low as 8.0 (Bijl et al., 2019).

The concentration of mineral chaperones can also be reduced by concentration of the milk by evaporation or reverse osmosis. For the standard milk composition, the concentration of mineral chaperones is zero after concentration by a factor of 1.5 at pH 6.7. At pH 6.5 or 6.3, it is zero for a concentration factor of 1.8 or 2.1, respectively. CaP also precipitates on heat exchanger surfaces during thermal treatments, sometimes producing the eggshell type of mineral rich deposit (Visser and Jeurnink, 1997, Abdallah et al., 2022). It is possible that the precipitation of CaP occurs to the greatest extent at the heat exchanger surface because sequestration of the CaP by the casein mineral chaperones cannot penetrate the protein layer that also forms at the surface (Singh et al., 2019).

AGE GELATION OF STERILIZED MILK

Gelation of concentrated protein solutions is often associated with amyloid fibril formation. Typically, this involves an initial rapid growth of amorphous

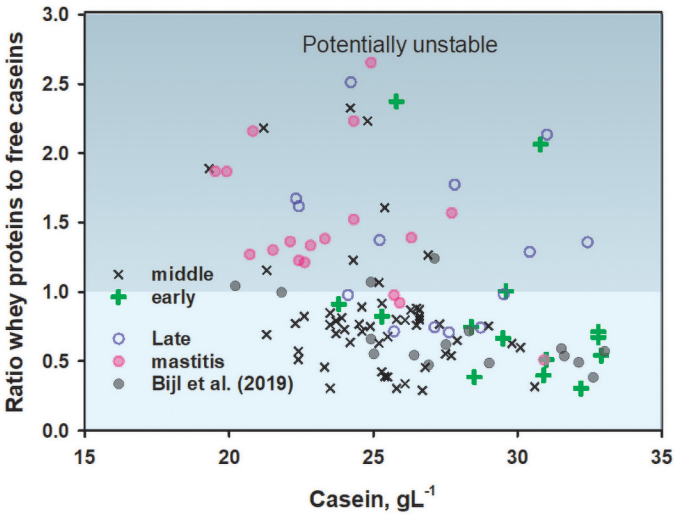

Figure 8. Stability of milk with respect to whey protein denaturation and aggregation during milk processing. Aggregation is inhibited by the total concentration of free casein molecular chaperones. Whey protein aggregation becomes more likely as the ratio of total whey proteins to free casein molecular chaperones increases. The sources for the data are described in Figure 6.

aggregates followed by a lag phase during which slow primary nucleation of fibrils takes place, mainly in the concentrated conditions of the amorphous aggregates. Amyloid fibril formation accelerates as the primary fibrils act as sites of secondary nucleation to rapidly increase the viscosity of the solution, leading to gelation shortly thereafter (Zhang, 2018, Phan and Schmit, 2022). These general ideas about amyloid fibril-induced gelation can be used to understand better the age gelation of UHT milk.

Figure 10 give a schematic overview of the physico-chemical aspects of age gelation. The thermally-induced unfolding and aggregation of cow β-Lg, shown in the top row of Figure 10, proceeds through a series of overlapping, partially unfolded intermediates of increasing disorder, as reviewed in more detail by (Sawyer, 1969, Sawyer, 2013, Cao and Mezzenga, 2019, Anema, 2021). At 80°C, the diversity of conformations is a maximum but there is a high degree of reversibility on cooling (Venturi et al., 2023). According to a study of β-Lg unfolding and aggregation by urea at pH 7 and 37°C (Hamada and Dobson, 2002), amyloid fibril formation required 10–30 d with the most efficient production occurring at 5-M urea. This urea concentration corresponds to the mid-point of the denaturation curve where there is the maximum concentration of partially unfolded β-Lg intermediates with intact cystine bridges, favoring amyloid fibril formation over disulfide interchange reactions (Hoppenreijs et al., 2023).

As shown in Figure 10, exposure of Cysteine, 121 and cystine bridges, particularly Cys 66-Cys 160 in β-Lg, allows disulfide interchange to occur with itself and κ-casein, resulting in a heterogeneous mixture of disulfide-linked oligomers and larger amorphous aggregates or strands, depending on the heat treatment, pH, and other environmental conditions (Heertje, 2014). Heat treatments rapidly cause casein micelles to become larger and depleted in free caseins, particularly κ-casein (Aoki and Imamura, 1975, Kudo, 1980) and to adopt a coarser internal structure with void spaces. Some of the smaller disulfide-linked oligomers of β-Lg and free caseins become associated with the micelles (Figure 10). According to the reviews of (Harwalkar, 1992, McMahon, 1996, Datta and Deeth, 2001, Deeth and Lewis, 2016), fibril strands are formed by co-aggregation of β-Lg and κ-casein, as subsequently confirmed immunologically and by fluorescence microscopy using different fluorescent labels attached to each protein (Dubert-Ferrandon et al., 2006, Malmgren et al., 2017, Raynes et al., 2017). Filamentous appendages to casein micelles and bridging filaments have also been observed by electron microscopy following forewarming of milk at 95°C for 10 min. or sterilization at 121.7°C for 15 min. (Davies et al., 1978) and in gelled UHT milk after storage for 34 mo at 5°C (Andrews et al., 1977). Growth and entanglement of the filaments is postulated to be a physico-chemical cause of age gelation in UHT milks (McMahon, 1996, Datta and Deeth, 2001, Deeth and Lewis, 2016). According to a recent study (Anema, 2021), none of the measured chemical or physical properties of the studied samples predicted which would form a gel. However, severe sterilization treatments to inactivate heat-stable proteinases such as plasmin is usually sufficient to postpone the onset of age gelation beyond the shelf-life of the product (Anema, 2019, Anema, 2022)

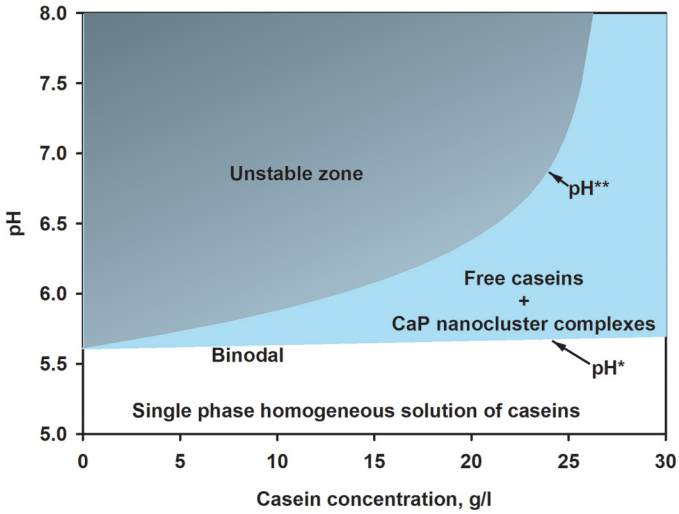

Figure 9. Stability diagram for a standard cow milk (Bijl et al., 2019). Below the critical pH, pH*, which lies on the binodal, the caseins form a single-phase homogeneous solution. Above pH*, the solubility of CaP is exceeded so a dispersed condensed phase of CaP nanocluster complexes is formed in equilibrium with a solution containing the excess concentration of free caseins. Further association of the complexes produces a polydisperse distribution of casein micelles, according to the multivalent-binding model (Holt, 2021, Holt and Carver, 2022). or, nearly equivalently, a dispersed condensed phase (Horvath et al., 2022). If pH is progressively increased at a fixed casein concentration, a point may be reached, pH**, where there are not enough casein mineral chaperones to sequester the CaP nanoclusters. As pH increases further, they become larger, less stable, and may eventually form a macroscopic CaP phase (Lenton et al., 2020).

Undisturbed storage of some UHT milk samples results in a compact sediment of depleted micelles and a cream layer (if fat is present). After some months, an increase in viscosity is apparent and is soon followed by the formation of a voluminous gel, beginning in the sediment layer then growing into the remaining volume. Although the sedimented layer is depleted in the fibril-forming β-Lg and κ-caseins, the high concentrations in the compact sediment lend themselves to the initiation of fibril growth.

An alternative mechanism of age gelation is that casein micelles that are depleted in free caseins are able to

aggregate in the sediment and then form the voluminous gel above the sediment layer (Anema, 2017, Anema, 2022). During the several months of storage preceding gelation, the samples that gelled also contained substantial hydrolyzed caseins. The composition of the residual whole casein in the sediment and voluminous gel was relatively depleted in κ-casein and enriched in β- and α_S-caseins compared with the starting milk. However, this compositional change occurred immediately after the heat treatment and did not change substantially thereafter. Moreover, the change of composition was similar in samples that did gel to those that did not. Proteolysis by plasmin or other proteases complicates the picture because the aggregation and fibril formation of the generated peptides could affect the onset and course of gelation.

Although no relationship has been found between gelation onset and the composition of the starting milk, this may be because the important variable identified in the multivalent-binding model is the ratio of the total concentrations of amyloidogenic proteins and peptides to the total concentration of molecular chaperones. The magnitude of this variable can be derived from the total milk composition but only after modeling or measuring the partition of salts and caseins (Bijl et al., 2019).

EFFECT OF EMULSIFYING SALTS

Emulsifying salts used in milk processing include sodium citrate and ortho-, meta-, pyro and poly-phosphates). They are used as additives, singly or in various combinations, to increase the stability of UHT milk and other milk protein products (de la Fuente, 1998). They can also act, in combination with calcium ions, as non-covalent cross-linking agents (Mizuno and Lucey, 2007). There is usually an optimum level of addition of the salts (Lewis, 2022). For example, in a study of processed cheese manufacture, the addition of pentasodium triphosphate led to casein phase separation, amyloid fibril formation of κ-casein and an increase in viscosity of the cheese mass (Vollmer et al., 2021a). Polyphosphates have been used to control the phase separation of insulin (Bye and Curtis, 2019), protamine (Kim et al., 2019) and histatin (Lenton et al., 2021) probably by binding to Lys and Arg residues and increasing the net negative charge of acidic proteins or decreasing the net positive charge of the basic proteins. When polyphos-

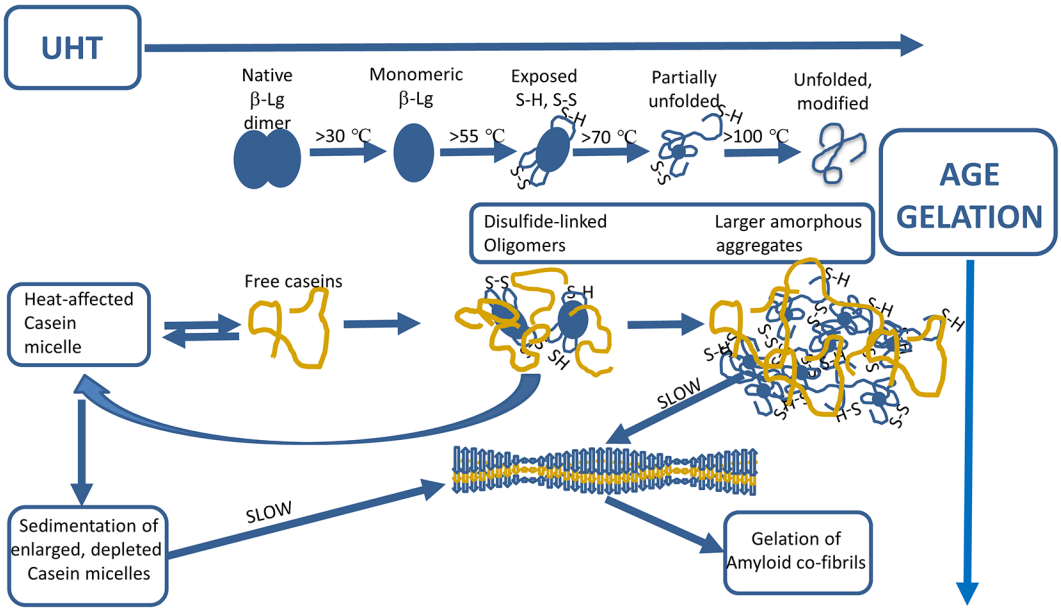

Figure 10. Heat treatments involving raised temperatures for different times-induce dissociation from the dimeric state and unfolding of the principal whey protein in cow milk, β-Lg. Unfolding proceeds in steps of increasing disorder with loss of helix and sheet secondary structure to give a range of partially unfolded intermediates. Exchange of micellar free caseins with the serum is also increased by higher temperatures. Limited aggregation and disulfide interchange of partially unfolded whey proteins produces some lower molecular weight, disulfide-linked, oligomers, limited in their growth by the molecular chaperone action of the free caseins. The oligomers, like the free caseins, exchange with the micelles. Larger amorphous aggregates are also formed rapidly by multivalent interactions of partially unfolded whey proteins and are stabilized by the free casein chaperones. Monomeric caseins and partially unfolded whey proteins can dissociate from the amorphous aggregates to slowly form co-fibrils, particularly between β-Lg and κ-casein. Age-thickening and gelation of the milk can occur during prolonged storage either by the slow growth of a network of the amyloid fibrils formed by the proteins or proteolytic fragments, or by the aggregation of depleted casein micelles that have reduced stability due to the net transfer of free caseins to the serum.

phates were added to a solution of α-synuclein, they promoted the formation of amyloid fibrils, possibly by cross-linking the peptide (Yamaguchi et al., 2021). As well as binding to proteins and modulating their net charge, all the emulsifying salts are chelators of calcium ions and this property has been incorporated into a model of the ion equilibria in milk serum (Gao et al., 2010). Nevertheless, despite their importance to milk protein technology, the binding strength and mode of interaction of emulsifying salts with milk proteins are not known.

CONCLUSIONS

Four different types of milk protein instability have been described and analyzed, i.e., the effects of salts, pH, whey protein destabilization, and age gelation, together with a non-classical mode of CaP precipitation. A significant factor controlling protein instability in milk and milk protein products is the total concentration of casein molecular chaperones available to prevent or limit amorphous aggregation or amyloid fibril formation by caseins or partially unfolded whey proteins. A significant factor preventing or limiting the precipitation of CaP in milk and milk protein products is the total concentration of casein mineral chaperones available to sequester the additional moles of CaP generated by concentration, pH increase, heat treatment or other processing operation.

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