Food Hydrocolloids 52 (2016) 670–677

Contents lists available at ScienceDirect

Food Hydrocolloids

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

Phase separation and composition of coacervates of lactoferrin and caseins

Skelte G. Anema [a, *], C.G. (Kees) de Kruif [b, c]

[a] Fonterra Research and Development Centre, Private Bag 11029, Dairy Farm Road, Palmerston North, New Zealand
[b] Van 't Hoff Laboratory for Physical and Colloid Chemistry, Debye Institute for Nano Science, Utrecht University, Padualaan 8, The Netherlands
[c] NIZO Food Research, PO Box 20, 6710 BA, Ede, The Netherlands

ARTICLE INFO

Article history:
Received 13 February 2015
Received in revised form
13 July 2015
Accepted 12 August 2015
Available online 14 August 2015

Keywords:
Lactoferrin
Casein
Coacervates
Composition
Salt

ABSTRACT

The bovine milk casein proteins (α_s-casein (ACN), β-casein (BCN) and κ-casein (KCN)) form complexes with lactoferrin (LF), an anionic protein also present in bovine milk that has an important bacteriostatic function especially in infant food. LF and each casein form complexes at near neutral pH, which is in between the respective pI's (≈8.3 and ≈ 4.6). The stoichiometry of the complexes is determined by the net charge of the proteins. Optimum complexation occurs at charge neutrality and is characterized by a maximum in turbidity. Mixing LF with each casein at a ratio where charge neutrality is obtained leads to a new complex coacervate phase. The kinetics of complex formation for LF/BCN and LF/KCN is rapid and appears to occur through a nucleation and coalescence process. However, the kinetics of complex formation between LF and ACN is much slower and therefore a nucleation and growth process is proposed, based on model calculations of the turbidity. The composition of the complexes is the same as the experimental mixing ratio if mixing ratio leads to a neutral complex. On standing and light centrifugation a complex-coacervate phase is formed, which is a viscous liquid.

1. Introduction

When two oppositely charged colloid particles interact, complex coacervation can occur where the colloidal system separates into two liquid phases. The coacervate phase is concentrated in the colloidal components whereas the remaining phase is the equilibrium solution (IUPAC, 1997; de Kruif, Weinbreck, & de Vries, 2004; Overbeek & Voorn, 1957; Voorn, 1956). Complex coacervation and phase separation of oppositely charged polyelectrolytes is well understood in terms of state diagrams (Bouhallab & Croguennec, 2014; Kayitmazer, Seeman, Minsky, Dubin, & Xu, 2013; de Kruif et al., 2004; Schmitt & Turgeon, 2011; de Vries, Weinbreck, & de Kruif, 2003; Weinbreck, Rollema, Tromp, & de Kruif, 2004). Complexes between oppositely charged colloids are usually formed if the pH is between the pI of the two polyelectrolytes and if the ionic strength is low. At higher ionic strengths, between 0.1 and 1 M, complexes are not formed indicating that the attractive interaction has both an electrostatic contribution and also a large and maybe dominant entropical component. The theory on complex coacervation recognizes these two contributions (Nakajima & Sato, 1972; Tainaka, 1979; Veis, 1961, 1963; Veis & Aranyi, 1960; Veis, Bodor, & Mussell, 1967; Voorn, 1956).

Most reports on complex coacervates are from studies on the combination of a polysaccharide and a protein under conditions where the macromolecules are oppositely charged. Examples are anionic gum Arabic and cationic β-lactoglobulin at low pH (Schmitt, Sanchez, Thomas, & Hardy, 1999; Weinbreck, Tromp, & de Kruif, 2004), puka gum and whey protein mixtures (Wee et al., 2014) or κ-carrageenan and β-lactoglobulin (Ould Eleya & Turgeon, 2000a, b). However, there are other systems as well, such as (synthetic) polymers and micellar systems. These different systems have been discussed and reviewed by de Kruif et al. (2004), Voets, de Keizer, and Cohen Stuart (2009), and Rawat and Bohidar (2014).

In contrast to polysaccharide/protein systems, studies on the complexation of two oppositely charged proteins are rather scarce (Bouhallab & Croguennec, 2014). There are examples of complex coacervation between the anionic casein proteins and cationic globular proteins such as lactoferrin (LF) or lysozyme (Anema & de Kruif, 2012a; Anema & de Kruif, 2013a) or between anionic

* Corresponding author. Fonterra Research and Development Centre, Private Bag 11029, Palmerston North 4442, New Zealand.
E-mail address: skelte.anema@fonterra.com (S.G. Anema).

http://dx.doi.org/10.1016/j.foodhyd.2015.08.011
0268-005X/

osteopontin and cationic LF (Yamniuk, Burling, & Vogel, 2009). In some respects the caseins and osteopontin are similar to the polysaccharides in that they are relatively unstructured and thus could be viewed as poly-ampholytic and poly-electrolytic macromolecules. In addition, the casein proteins can self-associate into polymeric micelle-like assemblies (Swaisgood, 1982, 1992). There are also some examples of coacervation between oppositely charged gelatins (Tiwari, Bindal, & Bohidar, 2009) or gelatin with casein (Milanović, Petrović, Sovilj, & Katona, 2014). Even more unusual is the phenomenon of complexation between two globular proteins. Examples include the association between LF and β-lactoglobulin (Anema & de Kruif, 2014), which appear to form stoichiometric (LF:β-lactoglobulin = 1:3) complexes independent of the pH, or the association between lysozyme and apo-α-lactalbumin, which forms complex coacervates as well as unique microspherical particles (Nigen, Croguennec, Madec, & Bouhallab, 2007; Nigen, Croguennec, Renard, & Bouhallab, 2007).

A few other examples of the association of LF with milk proteins have been reported. Ye, Lo, and Singh (2012) showed that, at pH 7, LF would associated with the proteins at the surface of oil-in-water emulsions stabilized by caseinate or whey protein isolate. This association was electrostatic in nature and reduced the magnitude of the zeta potential of the emulsion particles. The caseinate or whey protein stabilized emulsions were susceptible to flocculation by calcium; however, the association of LF with the caseinate or whey protein at the surface stabilized the emulsions against calcium-induced flocculation. Wang and Hurley (1998) showed that LF was present as high molecular weight complexes in mammary secretions during mammary gland involution. As caseins and whey proteins were also present in the high molecular weight complexes isolated from the secretions it was proposed that the LF formed complexes with these milk proteins, although LF self-association that formed high molecular weight LF oligomers was also a possibility.

In recent studies we examined the interaction of LF or lysozyme with the casein micelles in skim milk, and showed that the binding of these globular proteins to the casein micelles followed the Langmuir adsorption model (Anema & de Kruif, 2011, 2012b, 2013b). LF associated with the surface of the casein micelles whereas lysozyme interacted with both the surface and interior of the casein micelles. Interestingly, LF caused the disintegration of the casein micelles whereas lysozyme caused the aggregation of the micelles.

The complexation of LF and the caseins (pI ≈ 4.6) has been studied, initially by scattering/size measurements (Anema & de Kruif, 2012a) and subsequently by small angle x-ray scattering (de Kruif, Pedersen, Huppertz, & Anema, 2013). Complexes were formed at the pH between the respective pI's and at low salt concentrations. The stoichiometry of the complexes is determined by the net charge of the proteins and thus varies with pH. At each pH there is a maximum complexation if overall charge neutrality is reached. Away from this optimum ratio complexes do not coalesce due to the build up of extra charge.

In the previous work we investigated the equilibrium behavior of the complexes. Here we will present work on the kinetics of complexation on mixing LF with three different caseins (and at pH 6.55). The protein ratios were varied around the ratio where charge neutrality is reached. The optimum ratio is characterized by a maximum in turbidity and very low protein contents in the coexisting dilute phase. We investigated the kinetics of complexation by measuring turbidity, zeta potential, dynamic light scattering and a compositional analysis using electrophoresis of separated phases. We will try to establish whether complexes are formed by a nucleation and growth process or through coalescence of the initially formed complexes.

2. Materials and methods

2.1. Protein solutions

The ACN, BCN and KCN were obtained from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). LF (>90% purity) was supplied by the Fonterra Cooperative Group (Auckland, New Zealand). All proteins were used as supplied. Stock protein solutions of LF, ACN, BCN and KCN of ~10 mg/mL were prepared by mixing the proteins with water until dispersed. The protein solutions were filtered using syringe filters (0.4 μm) and then protein concentration in each filtered protein solution was determined using UV absorption at 280 nm and the known extinction coefficients for each protein (Swaisgood, 1982, 1992). Working solutions of ~1 mg/mL were prepared by accurately diluting the stock solutions with water. The pH of the working solutions was adjusted to pH 6.55 by the slow addition of 1 M HCl or 1 M NaOH while stirring.

2.2. Turbidity measurements

The transmission/turbidity of mixed LF and casein solutions were measured at a wavelength of 900 nm and in 1 cm path length cuvettes using a Jasco V580 spectrophotometer (Japan Spectroscopic Co., Hachioji City, Japan). All pure protein solutions had transmissions close to 100% in both water and the salt solutions used.

2.3. Centrifugation and electrophoresis

The coacervates were separated from the protein solutions by centrifugation (~27,000 g, 25 °C, 1 h in a bench centrifuge), and the supernatants were collected. The level and composition of protein in the original solutions and their respective supernatants were determined by microfluidic chip sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as has been described previously (Anema, 2009). For ease of analysis, the solutions were diluted in SDS-PAGE sample buffer so that a constant concentration of casein and increasing concentrations of LF were in the samples used for electrophoresis.

2.4. Zeta potential measurements

The zeta potential of the particles in the mixed protein solutions were measured using a Malvern Zetasizier nano ZS instrument and disposable folded capillary cells (Malvern Instruments, Malvern, Worcestershire, U.K.) using the techniques described previously (Anema & de Kruif, 2012b; Anema & Klostermeyer, 1996). Only samples in the turbid region were monitored and no further dilution or sample preparation was performed. The zeta potential measurements also provided the conductivities of the solutions.

2.5. Dynamic light scattering measurements

Dynamic light scattering (DLS) experiments were performed in manual mode and at a back scattering angle of 173° using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, Worcestershire, U.K.) and disposable plastic cuvettes. The details and methodology of this technique have been described previously (Anema & Li, 2003).

3. Results and discussion

3.1. Mixing LF and caseins

LF at a concentration of ~1 mg/ml was titrated into casein