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Influence of pH value and locust bean gum concentration on the stability of sodium caseinate-stabilized emulsions

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Abstract

Sodium caseinate emulsions of 20% sunflower oil in water were prepared at various pH and Locust Bean Gum concentrations (LBG). The presence of LBG was examined to assess the stabilizing properties in relation to flocculation, creaming and coalescence, in the initial emulsions as well freeze-thawed and freeze-dried/reconstituted samples. We found the initial emulsions to be stable at pH 6 and 6.5, both in absence or presence of LBG, against creaming. However we found evidence for the presence of emulsion droplet aggregates in the presence of LBG. Strong shear thinning behavior, even at low shear rates, and micrographs indicated that the presence of LBG may promote flocculation by mediating depletion forces between the oil droplets. However, the absence or low concentrations of LBG resulted in creaming followed by the emulsion break-up for the freeze-dried/reconstituted emulsions, particularly at lower pH. We have also detected a proliferation in the number of very small sub-micron particles in the particle size distribution, for samples containing higher concentrations of LBG, following the freeze-thaw cycle. We believe these to be LBG aggregates nucleating during the freezing phase of the process as the polysaccharide solubility declines with temperature.

Keywords: Emulsions; Locust bean gum; Sodium caseinate; Shear thinning; Freeze-drying

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1. Introduction

An emulsion is a heterogeneous system comprising of a dispersion of two immiscible liquids in which small droplets of one the liquids are homogenously dispersed throughout the other one, i.e. the continuous phase. Emulsions often become destabilized through a variety of different mechanisms including creaming, aggregation, coalescence and Ostwald ripening. To slow down all or some of these processes emulsifiers and stabilizers are used in the formation and subsequent kinetic stabilization of food emulsions. Emulsifiers are referred to as surface-active agents and possess both hydrophilic and hydrophobic sites within their molecules. These surface active materials promote the formation of the emulsion by lowering the interfacial tension due to their adsorption at the interface and provide a protective film between the two immiscible liquids (Hill, Ledward, & Mitchell, 1998; Sikorski, 2001) that enhances the short term stability of the droplets through the so called Gibbs-Marangoni effect (Dickinson, 1992).

Like emulsifiers, many proteins are surface active molecules and possess emulsifying properties. Some proteins are capable of adsorbing to the interface forming a charged protective layer around the oil droplets, and consequently retarding the flocculation and creaming rate by providing steric and electrostatic repulsion (Friberg, Larsson, & Sjoblom, 2003; Sikorski, 2001). The determining factors affecting the emulsifying properties of proteins encompass the presence of hydrophobic fragments on the surface of protein (a prerequisite for adsorption of a protein to the surface of the oil droplets), its charge, size and steric properties, cross-linking ability, solubility in aqueous phase, chain flexibility and the rigidity of protecting layers formed by the protein molecules (Sikorski, 2001).

Milk proteins are widely used as emulsifying agents in both soluble and dispersed forms in the food industry, since these ingredients show excellent surface-active and emulsion stabilizing characteristics (Dickinson, 1999). Casein, a protein derivative of milk, is commonly used as an emulsifier in oil-in-water systems. Due to the lack of an ordered structure, casein has a considerable number of exposed non-polar residues which facilitates its rapid absorption to the surfaces of newly formed oil droplets during homogenization. Furthermore, caseins form a thick protective layer of up to 10 nm around the oil droplets as compared with other milk proteins, e.g. whey proteins with only a layer of thickness 1-2 nm (Surh, Decker, & McClements, 2006). The thick interfacial layer confers strong and long ranged steric repulsion on the freshly formed oil droplets and protects fine droplets against flocculation and hence coalescence (Dickinson, 1999; Surh, et al., 2006). Caseinate (CAS) covered emulsions are more stable to elevated temperature treatments when compared with whey protein stabilized emulsions since in contrast to the globular proteins, e.g., whey proteins, casein molecules are relatively flexible and do not develop heat-induced structural changes (McClements, 2004; Surh, et al., 2006). One drawback of CAS-stabilized emulsion is their susceptibility to droplet flocculation at pH close to the isoelectric point (pH 4.6) of the casein since this drastically reduces the electrostatic repulsion between the oil droplets (Perrechil & Cunha, 2010; Surh, et al., 2006).

A variety of oil-in-water emulsions, e.g., frozen foods and pharmaceuticals, are preserved at low temperature to improve their shelf life against microbial, enzymatic, and chemical deterioration. Upon freezing and chilling, oil-in-water emulsions are significantly destabilized, and then readily broken down after thawing (McClements, 2004). Deep under-cooling of emulsions may induce crystallization, and consequently the formation of a fat crystal network in the oil droplets. Some of these crystals may protrude from the fat globules and disrupt the surrounding protective film which results in the formation of rigid granules. This phenomenon is called partial coalescence in which the granules cannot form a larger droplet due to the crystal network in the fat globules. However, upon melting of the fat crystals at sufficiently higher temperatures, i.e. thawing, rigid granules are gradually transformed into larger droplets with coalescence now progressing to completion (Walstra, Wouters, & Geurts, 2005).

Drying technologies are also used to enhance the shelf life of oil-in-water emulsions. The resultant dried emulsions either can be easily used as food ingredients, e.g., flavoring agents, or can be reconstituted with water by the consumer, e.g., dairy creamers (McClements, 2004). There is a growing interest in freeze drying of emulsions in the pharmaceutical industry since the dried emulsions are easy to

handle and physically stable ingredients (Christensen, Pedersen, & Kristensen, 2002). During the dehydration step of freeze drying process, the removal of water from the protein may lead to a loss of protein functionality and stability (McClements, 2004).

In the context of food colloids, the term stabilizer is often defined as a component having sufficient capacity to stabilize emulsions (Hill, et al., 1998). The addition of polysaccharide as a stabilizer in oil-in-water emulsions not only improves the textural properties but also increases the viscosity of the aqueous phase. This slows down the rate of creaming (McClements, 2000). It also arrests the Brownian motion of the droplets, thus preventing their collision and coalescence. Locust bean gum (LBG) is an important thickening polysaccharide composed of a main backbone of 1-4-linked β-D-mannopyranosyl units, with 1-6-linked α-D-galactopyranosyl units. When LBG suspension is heated around 85˚C , most particles swell, producing a high viscous solution with some degree of thixotropic properties (Whistler & BeMiller, 1997). Locust bean gum is compatible with other food polymers, widely used in dairy and frozen dessert products, due to the neutral character of this hydrocolloid (Everett & McLeod, 2005; Whistler, et al., 1997).

In addition, this neutral galactomannan gum has also been reported to possess emulsifying properties at a relatively low LBG/oil ratio (1:5) by lowering the interfacial tension due to a peptide fraction tightly bound to the polysaccharide (Dickinson, 2003). Wu, Cui, Eskin & Goff (2009) studies and contrasted the emulsion capacity and stability for several different gums/. He found the emulsifying property of LBG to be lower than fenugreek gum , guar gum and tara gum. There has also been some studies that have suggested that locust bean gum is not surface active and that the surface activity is the result of the presence of impurities in the LBG used (Gaonkar, 1991). Nevertheless, in the present study LBG has to compete with the significantly more surface active sodium caseinate for adsorption onto the surface of oil droplets. CAS is known to be able to displace many other proteins from the interface(Dalgleish, 2011). In this respect the behavior of CAS is akin to that of smaller molecular weight emulsifiers (surfactants). Due to their much better packing on the surface, surfactant molecules are efficient displacers of larger amphiphilic macromolecules from the air-water and oil-water interfaces (Pugnaloni, Dickinson, Ettelaie, Mackie, & Wilde, 2004). Thus, in presence of a sufficient amount of CAS it is unlikely that the much larger molecule such as LBG, comprising largely of hydrophilic polysaccharide, can directly adsorb to the interface. If there is to be any adsorption of LBG this would have to be in the form of a secondary layer adsorbed on top of the primary caseinate layer. Adsorption of polysaccharides to hydrophobic-hydrophilic interfaces in this manner has been investigated both experimentally (Guzey & McClements, 2006; Guzey & McClements, 2007; Jourdain, Schmitt, Leser, Murray, & Dickinson, 2009) and through numerical theoretical calculations (Ettelaie, Akinshina, & Dickinson, 2008; Ettelaie, Akinshina, & Maurer, 2012). These studies have shown the need for strong favorable interactions between protein and polysaccharides before the formation of a secondary adsorbed layer by the latter can commence. The favorable strong interaction between protein and polysaccharide are requires in order to compensate for the loss of configurational entropy incurred by the polysaccharide, upon its adsorption on the interface. Such interactions can arise from the opposite electrical charge of proteins and polysaccharide at low pH values, below pI of the protein. Locust bean gum lacks the necessary charge to form such complexes with the protein, even if one overlooks the fact that our studies are all performed at pH values above the isoelectric point of caseinate. In fact, there are even some studies that suggest that there might be some degree of incompatibility between LBG and CAS (Vega, Dalgleish, & Goff, 2005). Therefore, we expect that a large fraction, if not all of the LBG to remain in the bulk solution in the emulsion systems investigated in this work. The point is highlighted here since it is a rather important consideration in the discussion that will fellow when interpreting our experimental results.

To the best of our knowledge, only a few studies so far have been conducted to specifically investigate the influence of LBG ratio on the stability of CAS-stabilised emulsion. Perrechil and Cunha (2010) investigated the influence of LBG on the stability of neutral emulsions stabilized by sodium caseinate during the storage at room temperature. Furthermore, Perrechil, Braga & Cunha (2009) studied the effect of LBG concentration on the microstructure and rheological properties at the protein isoelectric point of sodium caseinate.

The aim of this study was to investigate how pH and LBG concentration would influence the stability of CAS-stabilized emulsion during aging, freezing, and freeze drying.

2. Materials and methods

2.1. Materials

Casein was purchased from Arla Foods Ingredients Group (Leeds, UK), and locust bean gum (LBG) was obtained from Agrisales (Agrisales Ltd, Bridgewater, UK). Refined sunflower oil was purchased from a local supermarket. The different buffer solutions were made with de-ionized water.

2.2 Solution preparation

Sodium caseinate solutions (1.0 wt%) were prepared by adding the casein powder to buffer solutions, stirring with magnetic bar for 60 min at room temperature. Na-phosphate buffer solutions were made for pHs 6.0, 6.5, and Na-acetate buffer solutions were made for pHs 5.0 and 5.5. LBG solutions (0.2, 0.4, 0.6 wt%) were prepared by dissolving the powder in buffer solutions, stirring and heating the LBG dispersions at 85˚c for 10 min to dissolve the polysaccharide entirely. The resultant LBG solutions were immediately cooled in a water bath at 17±1˚C. Both pure casein and polysaccharide solutions were mixed and stored in a refrigerator overnight to fully hydrate the hydrocolloids.

2.3 Emulsion preparation

All oil-in-water emulsions containing 20 vol % sunflower oil and 80 vol % aqueous emulsifier solution (1.0 wt % sodium caseinate, and different LBG concentrations) at different pH values, i.e., 5.0, 5.5, 6.0, and 6.5, were prepared using a high-pressure jet homogenizer (Burgaud, Dickinson, & Nelson, 1990) operating at a constant pressure of 200 bar.

2.4 Freeze drying of emulsions

20 ± 1 g of each emulsion was poured into a Petri dish (internal diameter 86.4 mm), then stored in a freezer for 24 h at -20±1˚C. The frozen samples dried with a laboratory freeze dryer (S B Freeze Driers Limited, Folkestone, UK) to a constant vacuum pressure of 4 torr.

2.5 Creaming stability measurement

After emulsion preparation, 30 ml of each emulsion was transferred into two glass test tubes (internal diameter 23.5 mm, height 100 mm), and then sealed with a plastic cap. One of the glass test tubes was stored at room temperature for 10 days (with sodium azide preservative) and another test tube was stored in a freezer for 24 h at -20 ± 1˚C, followed by thawing emulsion in a water bath at 17±1˚C. After both freeze-thaw treatment and aging, i.e., the storage of emulsions for a period of time at room temperature, some of emulsions were separated into different phases, i.e. an apparent cream layer at the top of the test tube and a serum layer at the bottom. The total height of emulsion (H_E) and the height of serum layer (H_s) were measured using a ruler and a creaming index was reported as (%) =100(H_s/H_E).

2.6 Particle size measurement

The particle size of CAS-coated emulsion droplets was measured using a laser light scattering instrument (Mastersizer, Malvern Instruments Ltd, Worcestershire, England, UK). Before particle size measurement, emulsions were completely mixed. Sample was added to the dispersion unit connected to the laser light scattering instrument until an obscuration between 10% and 12% was reached. The mean particle size was reported as the volume-weighted mean diameter, ($d_{43} = \sum \mathrm{n_i}\, d_i^4 / \sum \mathrm{n_i}\, d_i^3$), where n_i is the number of particles with diameter d_i. The frozen emulsions were thawed in a water bath at 17±1˚C and then gently shaken before measurements and freeze-dried emulsions were reconstituted by adding the dry emulsions to distilled water, stirring with magnetic bar for 60 min at room temperature. Also emulsion

droplets were visualized using an optical microscope (Bresser LCD Micro, Germany). Emulsion droplets were placed directly onto a glass microscope slide and viewed under 400X magnification.

2.7 Rheological measurements

The influence of pH and LBG concentrations on the stability of CAS-coated emulsion droplet can be further investigated using rheological measurements. The apparent viscosity of emulsions was measured 24 h after emulsion preparation using a Bohlin C-VOR rheometer (Malvern Instruments Ltd., Worcestershire, England, U.K.), with a C25 cup and bob geometry. The sample was gently mixed, after 30 min poured into the temperature-controlled measurement cell, and allowed to equilibrate at 25 ℃ for 10 min prior to the measurement. Apparent viscosity of emulsions was measured at shear-rates in the range 0.1–200 s^{-1} using continuous shear, with a 30 s delay time and a 30 s integration time at 25 ℃. Viscosity measurement was only performed for visually non separated emulsions after gentle mixing. No measurement was carried out for frozen and freeze-dried emulsions. In particular, this excludes emulsions with no, or small concentration of LBG at lower pH values of 5 and 5.5.

3. Results

3.1 particle size measurement

After emulsion preparation, particle size measurement of most CAS-stabilized emulsions indicated a reduction in the volume-weighted mean droplet diameter (d_{43}) with increasing pH value and LBG concentration. All oil-in-water emulsions at pH 5.0 exhibited a larger mean diameter (d_{43}) as compared with other pH values. The maximum d_{43} value (143 μm) was found at pH 5.0 in the absence of LBG. At any given value of pH, the higher concentrations of the polysaccharide significantly decreased the mean particle size. The reduction was particularly pronounced at pH=5, where the average size decreased from 143 μm in absence of LBG to 64 μm in presence of 0.6% locust bean gum. At higher pH values above 5.0, the change in the size of the droplets with the concentration of LBG is less dramatic. In fact for emulsions prepared at pH 5.5 and 6.5, and containing 0.2% LBG, d_{43} values of 31 μm and 10 μm were measured respectively. These were slightly higher than those for the corresponding systems in the absence of LBG (Fig. 1). Thus it seems that at higher pH values, away from pI, the size of droplets is not a strongly dependent function of locust bean gum concentration in our emulsions.

Particle size measurement of emulsions after freeze-thaw treatment revealed a new range of mean droplet diameter (d_{43}). The influence of pH and LBG concentration on the particle size distribution of initial emulsions was similarly observed after freeze-thaw treatment with a new range of d_{43} values. Most emulsions in the absence of thickening agent or in presence of 0.2% LBG exhibited an increased d_{43} value as compared with the initial oil-in-water emulsions. The maximum value in d_{43} (173 μm) was once again found at pH 5 without LBG as a thickening agent. Surprisingly, some thawed emulsions containing higher LBG concentrations indicated a slightly smaller d_{43} when compared with those for the initial emulsions (Fig. 2). However, the reduction in the value of d_{43}disguises some important changes in the emulsion system that are otherwise only revealed by a more careful examination of the entire particle size distribution. Figures 3a, 3b and 3c show the distribution of particle sizes obtained by the Mastersizer at pH=6.5, prior to freezing, following freeze-thawing and after freeze drying and reconstitution, respectively. Each figure contains the graphs for systems with LBG concentrations of 0.0%, 0.2%, 0,4% and 0.6%. The size distributions for the freeze-thawed samples at higher LBG concentrations of 0.4 and 0.6% show evidence for the presence of small particles in the size range 0.3-1.0 μm, previously not present in the original emulsions (Fig. 3a and b). It is the formation of these that is mainly responsible for the observed downward shift in the value of d_{43}following the freeze-thawing cycle. The small particles are not formed for emulsions containing no or low (0.2%) concentrations of LBG. This provides some clues as to the origin of these particles. We shall discuss this point later in section 4. Similar results are also observed at pH=6, although the presence of small particles following freeze-thawing is slightly less pronounced than that for pH=6.5 systems.

The Stability of oil-in-water emulsions was greatly altered by freeze drying process. At pH 5.0 and LBG<0.6 wt%, all CAS-stabilized emulsions underwent a pronounced destabilization and separated into

distinct oil and solid phases. After reconstitution of freeze-dried emulsions, the particle size distribution was determined in a range of 61-170 μm. In all reconstituted emulsions, the lowest LBG concentration (0.2 wt %) displayed the largest increase in the size of CAS-coated emulsion droplet. However at higher LBG concentrations, d_{43} continued to decrease (Fig. 4).

3.2 Creaming stability measurement

In these series of experiments, the influence of pH and LBG concentration on the stability of oil-in-water emulsions to the gravitational separation was evaluated during storage at room temperature and freeze-thaw treatment. After 10 days of storage, observational evaluation of emulsions indicated that most CAS-stabilized emulsions were separated into an apparently opaque cream layer at the top of the test tube and a transparent serum layer at the bottom. Emulsions prepared at pH 5.0, i.e., close to the protein's isoelecteric point, exhibited the greatest susceptibility to gravitational separation. CAS-stabilized emulsions at pH 5.0, in the absence of LBG illustrated the highest value of creaming index up to 50%. With Increasing pH and LBG concentration, a substantial decrease in creaming index was observed in most cases. The presence of thickening agent at the concentration above 0.2% LBG indicated a more pronounced effect on the stability of oil-in-water emulsions. CAS-stabilized emulsions at pH values above 5.0, containing 0.6% LBG exhibited a high resistance against creaming, and no gravitational separation was observed after 10 days of storage (Fig. 5).

After freeze-thaw treatment of emulsions, most samples were visually observed to separate into different layers, i.e., a top layer of free oil, an intermediate opaque layer of cream and a transparent serum layer at the bottom of the test tube. The stability of oil-in-water emulsions to the freeze-thaw treatment was greatly influenced by the pH value and LBG concentrations. Like emulsions stored at room temperature, all oil-in-water emulsions at pH 5.0 exhibited the greatest susceptibility to the gravitational separation. Free oil layer was only observed in CAS-stabilized emulsions at pH 5.0, in the absence and in presence of 0.2% LBG. Increasing pH value and LBG concentration in initial emulsions, resulted in a sharp decrease in creaming index, and CAS-stabilized emulsions at pH above 5.5 containing 0.6% LBG indicated no phase separation after freeze-thaw treatment of emulsions (Fig. 6).

3.3 Rheological measurement

Rheological measurements were carried out on emulsion samples that showed no obvious signs of break up after 24 hours of storage. For all cases tested, the systems exhibited a shear thinning behavior over the range of shear rates examined (0.1 to 200 s^{-1}). The same was also found by Perrechil and Cunha (2010) who highlighted that CAS-stabilized emulsions in the presence of LBG exhibited shear thinning behavior. The rheological data for all samples was described well by the equation

$$\sigma = K\dot{\gamma}^n$$

for a power law fluid, where, K is the consistency index ($Pa\ s^n$), the exponent *n* is the flow behavior index, $\dot{\gamma}$ the shear rate (s^{-1}) and σ the shear stress (Pa). The flow behavior index is a dimensionless number indicating the closeness of the fluid rheology to Newtonian flow, thereby characterizing fluids as Newtonian, shear-thinning, or shear-thickening fluids based on its magnitude. When $n = 1$, the viscosity of the fluid is shear independent and the fluid is referred to as a Newtonian fluid. For cases $n<1$ or $n>1$ the fluid is shear-thinning or shear-thickening, respectively. Two parameters K, i.e., consistency index ($Pa\ s^n$), and *n*, i.e., flow behavior index, were found to be strongly influenced by different pH values and LBG concentrations. The maximum value of consistency index and a strong shear thinning behavior was found at pH 5.0 and 0.6% LBG (Fig. 7 and Fig. 8). CAS-stabilized emulsions at higher PH values and lower LBG concentrations showed a noticeable reduction in the value of the consistency index, K(Fig. 7). Interestingly, in the case of 0.2% LBG concentration, consistency index was slightly lower (2.14 $Pa\ s^n$) in oil-in-water emulsions prepared at pH 5.5 when compared with those at pH 6.0 (2.50 $Pa\ s^n$).

Figures 9 and 10 show logarithmic plots of apparent viscosity $\eta = \sigma / \dot{\gamma}$ (Pas), vs. shear rate $\dot{\gamma}$ (s^{-1}) for our emulsions at pH=6 and pH=6.5, respectively. The linear variation of ln(η) with ln($\dot{\gamma}$), i.e. $\log \eta = \log K + (n-1) \log \dot{\gamma}$, is evident in all cases, but particularly noticeable for emulsions containing LBG. It is also clear from Fig. 9 and 10 that the flow behavior of emulsions that were prepared without LBG, but yet remained stable, is markedly less shear thinning than those that included locust bean gum. The two cases in mind are the emulsions at higher pH values were the flow behavior index was *n*=0.85 at pH=6.5 and *n*=0.79 at pH=6. The closeness of the value of *n* to 1, particularly for the pH=6.5 system, indicates that the flow behavior for these emulsions, in the absence of LBG and at sufficiently high pH, is only mildly shear thinning and not far off being Newtonian. In contrast, the same values for the corresponding systems with 0.2% and 0.6% were 0.31 and 0.27 at pH=6.5 and 0.29 and 0.29 for pH=6.0. This reflects the highly shear thinning behavior associated with the emulsions containing LBG.

4. Discussion

4.1 Particle size measurement

The evaluation of particle size distribution of CAS-stabilized emulsions, prior to freezing, revealed that the volume-weighted mean diameter (d_{43}) is affected by LBG concentration and both low and high pH values. All oil-in-water emulsions prepared at pH 5.0 exhibited a significantly larger d_{43} value as compared with the emulsions prepared at higher pH values. The maximum d_{43} value (143 μm) was found at pH 5.0 in the absence of LBG. The results suggest that at pH 5.0, oil droplets are very susceptible to droplet flocculation. This is expected since this pH value is quite close to the isoelectric point of adsorbed proteins (pH 4.6). The reduction in the charge of the protein, decreases the electrostatic repulsion between the droplets, allowing the attractive forces between them to dominate (Perrechil, et al., 2010; Surh, et al., 2006). In most cases, the higher concentrations of LBG as a thickening agent noticeably decreased the volume-weighted mean diameter (d_{43}). As discussed in the introduction, we believe that most, if not all of the LBG resides in the bulk, due to its competitive adsorption with the significantly more surface active CAS. The effect of a non-adsorbing polysaccharides, such as LBG, on the stability of droplets is two folds. On one hand such polysaccharides give rise to increasing viscosity of the aqueous phase between the dispersed oil droplets, consequently slowing down the Brownian motion of the droplets in the emulsion and thus reducing the collision rate between them (Dickinson, 2003; Hill, et al., 1998). On the other hand free polysaccharides can also induce attractive depletion forces between the droplets (Khalloufi, Alexander, Goff, & Corredig, 2008; Moschakis, Murray, & Dickinson, 2006), promoting the flocculation and creaming in the emulsion system. The attraction arises due to exclusion of non-adsorbing polysaccharides from the narrow gaps between CAS-coated emulsion droplets, due to their extended and stiff structure. The resulting osmotic pressure between the polysaccharide free gap region and polysaccharide solution outside is what induces the attraction between the droplets. However, such depletion interactions tend to be rather weak compared to other colloidal forces. Their addition to the already present net attractive forces between the droplets at pH=5.0, do not alter the nature of the inter-droplet forces substantially. In these cases then it is the stabilizing effect of LBG, through the increased bulk viscosity, that is expected to be the dominant factor. By preventing the formation of larger aggregates, they account for the observed decrease in the mean droplet diameter.

The above situation alters once we consider pH values away from pI. Oil-in-water emulsions prepared at pH 5.5 and 6.5, containing 0.2% LBG, showed slightly higher d_{43} values than those in the absence of LBG. Although the changes in the mean droplet diameter overall was found not to be as sensitive to the amount of added LBG, at these higher pH values (Fig. 1). With increasing non-absorbing polysaccharide concentration, the attractive depletion force between the oil droplets increases. At a specific concentration known as the critical flocculation concentration (CFC), the attractive force overcomes the repulsive force between the oil droplets and causes them to flocculate. However, at higher concentrations of polysaccharide, i.e., well above CFC, the flocculation rate decreases since the increased viscosity causes CAS-coated oil droplets to become immobilized as was discussed above (Dickinson, 2003; McClements, 2004). CAS stabilized emulsions prepared at pH values far from pI are

already quite stable, in the absence of LBG. For these, any changes brought about by the addition of LBG require a more careful examination of the entire particle size distribution rather than just a consideration of d_{43}.

Figure 3 display the entire particle size distribution for the emulsions prepared at pH=6.5. Several systems with different LBG content are shown. For the emulsions prior to freezing, it is noticed that the distributions have two distinct parts, with a second smaller peak occurring at a size of around ~ 100 μm, considerably bigger than the size of a few μm at which the first larger peak occurs. The second peak in the size distribution is shown more clearly in the inset in the figure. It is seen than in the presence of LBG, the number of these larger particles has increased and they also extend to larger sizes. The considerably larger size of the droplets in this second peak, as compared to primary oil droplets in the first peak, points to these either being aggregates of emulsion droplet or else large individual droplets formed by coalescence. While the second possibility cannot be entirely ruled out, based on our rheological data to be discussed below, we are inclined to believe that these larger particles are aggregated of many smaller individual emulsion droplets. This view is also supported by examining a selection of our emulsions under microscope. Figures 11a, 11b, display micrographs for the emulsion prepared at pH=6.5, without any LBG and with 0.2% LBG. Presence of a considerably more uniform structure in the first micrograph is evident. Addition of LBG has caused the formation of what look to be relatively open and ramified aggregates of emulsion droplets. We also examined the system of Fig. 3b after 30 min post dilution. The emulsion were diluted with water at a ratio of 10:1. The results are displayed in Fig. 11c. Although the remnants for the presence of aggregates are still just about detectable, these are smaller and the structure as a whole seems more uniform. This is in line with the behavior of depletion flocculated aggregates, though the time scales required for aggregates to fall apart, following dilution, do pose an interesting problem which merits future investigation. Finally, Fig. 11d shows the structure of emulsion after the freeze-thawing cycle for the 0.2% LBG system, at pH=6.5. Visual macroscopic inspection of the sample did show a considerable degree of creaming in this system. The presence of large compact aggregates (and possibly coalesced) drops in the micrograph, coupled with the lower amount of LBG and therefore bulk viscosity can account for the poor observed creaming stability of this emulsion. Large coalesced droplets are clearly visible in micrographs for emulsions with 0.0% and 0.2% LBG at pH=6.5, following freeze-drying and reconstitution (Fig. 11e and f).

The freeze-thaw treatment of most CAS-stabilized emulsions containing less than 0.4% LBG at pH values below 6.0, led to a noticeable increase of the mean droplet diameter (d_{43}) which is consistent with the creaming stability measurements and macroscopic observation of emulsions, indicating that the emulsions were destabilized during freezing and then broken down by thawing process. When oil-in-water emulsions are frozen to a temperature at which water crystallizes, a number of physicochemical processes occur which may lead to the destabilization of emulsion systems. Firstly, oil droplets are excluded from the frozen phase occupied by ice crystals and forced into close contact. Secondly, upon freezing, the amount of liquid water may not be sufficient to fully hydrate the emulsifier molecules which may cause interaction between the CAS-coated oil droplets. Thirdly, the freezing causes ions to become increasingly concentrated in the unfrozen phase, consequently this decreases the electrostatic repulsion between the oil droplets. Finally, the ice crystals may pierce the protective film around the fat globules. Deep under-cooling of the emulsion may induce crystallization and consequently the formation of a fat crystal network in the oil droplets. Some of these crystals may protrude from the fat globules and disrupt the surrounding protective film. A protruding crystal may penetrate into another partially crystalline fat droplet, and eventually lead to the formation of rigid granules. This phenomenon is called partial coalescence in which the granules cannot form a larger droplet due to the crystal network in the fat globules. However, upon melting the fat crystals at sufficiently high temperatures, i.e. thawing, rigid granules are gradually transformed into larger droplets. This process is called coalescence (Walstra, et al., 2005).

Particle size measurement of emulsions after freeze-thaw treatment indicated that higher locust bean gum concentrations improved the stability of CAS-coated emulsion droplet to the droplet aggregation since LBG may alter the physicochemical processes during freezing by controlling the amount of ice crystals formed, and thereby controlling the stability of oil-in-water emulsions (Goff, Caldwell, Stanley, & Maurice, 1993). However, surprisingly most thawed emulsions at pH values above

5.5 containing LBG> 0.2% exhibited a slightly smaller mean diameter (d_{43}) than those of the initial CAS-stabilized emulsions. The reason for this can be seen in Fig. 3b, showing the particle size distribution of oil-in-water emulsions post freeze-thaw treatment procedure. Emulsion stability can be affected by the collision rate of partially crystallized fat globules. In the limited time, the rate of collision between small partially crystallized fat droplets is higher than large droplets and may result in partial coalescence (Vanapalli, Palanuwech, & Coupland, 2002). Most CAS-stabilized emulsions at pH values above 6.0 indicated bimodal particle size distributions. For example for a typical CAS-stabilized emulsion at pH 6.5 containing 0.4% LBG, main volume of CAS-coated droplets was distributed in the range of 1 - 10 μm and a secondary particle population was observed in the range of 0.3 - 1 μm, following freeze-thawing. In this emulsion a narrow particle population was also found in the range of 0.02 - 0.3 μm. Immediately after thawing of oil-in-water emulsions, a significant increase in volume of small particles in a range of 0.04 - 1 μm was found which caused a shift of mean droplet diameter (d_{43}) toward smaller values (Fig. 3b). It is also noticeable that these small particles are only detectable for systems containing LBG. Since we do not expect the freeze thawing procedure to produce oil droplets smaller than those already present in the unfrozen sample, we conclude that these small particles are most likely to be aggregates of LBG. These would have been produced during the freezing phase as the solubility of the polysaccharide in the water began to drop with lowering temperature. At the same time, locust bean gum would be increasingly accumulated in the unfrozen water regions, pushing its concentration in these regions to higher values. At some stage then, the concentration of LBG will surpass its solubility limit causing phase separation of LBG by nucleating polysaccharide particle aggregates. These may take a long time to dissolve back in water following thawing at room temperature, rather than the elevated temperature used initially to dissolve the LBG in preparation of original emulsions. Excluding these small particles from the distribution produces a man droplet size that is now consistently greater than the original non-treated emulsion as one may expect.

Freeze drying process extensively destabilized CAS-stabilized emulsions (Fig. 3c). After reconstitution of freeze-dried emulsions, laser diffraction measurements exhibited a noticeable increase in the mean droplet diameter (d_{43}) of all emulsions. d_{43} value of all reconstituted emulsions substantially increased at 0.2% LBG and then decreased at higher LBG concentrations. The smallest increase in d_{43} was observed in emulsions containing 0.6% LBG at pH 6.5. The destabilization of emulsions might be due to the physicochemical processes occurring during both freezing, and drying steps. As discussed earlier, many factors affect emulsion stability during the stages of freezing. Similarly, during the drying process, proteins can be denatured due to the removal of water, thereby losing their functionality (McClements, 2004). This is less likely though with CAS, since this has already a coil like, disordered structure and therefore those not undergo denaturation (Dalgleish, 2011). A contributing effect to the increased d_{43} measure in the reconstituted emulsions containing LBG can be the depletion flocculation induced by non-adsorbing polysaccharides, i.e. locust bean gum, which may have occurred in the initial emulsions or during reconstitution of the freeze-dried emulsions. In the latter case, the small particles formed by LBG during freezing may themselves contribute to depletion flocculation of larger droplets, as well as the fraction of LBG dissolved in the bulk as individual macromolecules. Once again this effect has to be balanced against the stabilizing effect of LBG at higher concentrations due to immobilizing the droplets and the increased viscosity (McClements, 2004). In addition, LBG at higher concentration may also alter the physicochemical processes by controlling the amount of ice crystals and modifying the unfrozen phase during the freezing step and by providing a viscous glass layer around the CAS-coated oil droplets during the dehydration step (Goff, et al., 1993; McClements, 2004).

4.2 Creaming stability measurement

After 10 days of storage, most CAS-stabilized emulsions were separated into an apparently opaque cream layer, i.e., creamed layer, at the top of the test tube and a transparent serum layer, i.e., droplet-depleted layer, at the bottom.

All oil-in-water emulsions at pH 5.0 exhibited a greater degree of creaming when compared with emulsions at higher pH values. The results suggest that CAS-stabilized emulsions prepared at pH 5.0 are extremely susceptible to the gravitational separation. The reason for this can be explained by the fact that at pH value very close to the isoelectric point of adsorbed proteins (pH 4.6), the net attractive forces

between casein molecules increases, leading to the decreased electrostatic repulsion, and consequently CAS-coated oil droplet aggregation (Perrechil, et al., 2010; Surh, et al., 2006). The droplet aggregation, e.g., flocculation and coalescence, may enhance creaming rate by increasing the oil droplet size (McClements, 2005). The creaming index values are consistent with the experimental laser diffraction measurements, showing a significantly larger d_{43} value for all oil-in-water emulsions at pH 5.0. Most cases indicated a substantial decrease in creaming index at higher pH values and LBG concentrations. LBG give rise to an increase in viscosity of the aqueous phase which consequently slows down the movement of oil droplets in the emulsion (Dickinson, 2003; Hill, et al., 1998). However, interestingly oil-in-water emulsions prepared at pH 6.5 containing 0.2% LBG, showed a higher creaming index when compared with those in the absence of a thickening agent. This phenomenon can be attributed to depletion flocculation induced by non-adsorbing polysaccharides, e.g., locust bean gum, at the critical flocculation concentration (CFC) (as discussed in earlier sections).

After freeze-thaw treatment, emulsions were separated into different layers, i.e., a top layer of free oil, an intermediate opaque layer of cream and a transparent serum layer at the bottom of the test tube. Macroscopic observation of thawed emulsions suggested that oil-in-water emulsions were destabilized and then broken down through freeze-thaw treatment. The formation of free oil layer at the top of the test tube is referred to as oiling off, resulting from the coalescence of oil droplets during freeze-thaw processes as suggested by McClements (2004, 2005).

The stability of oil-in-water emulsions to the freeze-thaw treatment was greatly influenced by the pH value and LBG concentrations in the initial emulsions. Increasing pH value and LBG concentration, resulted in a sharp decrease in the creaming index, and CAS-stabilized emulsions at pH above 5.5 containing 0.6% LBG indicated no phase separation after freeze-thaw treatment of emulsions. These results are in accordance with the light scattering data of the thawed emulsions. The destabilizing effects of freezing and the influence of locust bean gum concentration on the stability of emulsions were specifically discussed in earlier sections.

4.3 Rheological measurements

Behaviour of all emulsion systems, prior to freezing, followed a power law fluid rather well (e.g. see Fig. 9 and 10). Evaluation of the rheological properties of the initial emulsions, based on the power law model indicated that two parameters K, i.e., consistency index ($Pa\ s^n$) and n, i.e. flow behavior index, were both greatly influenced by different pH values and LBG concentrations. Most emulsions indicated an increased apparent viscosity at low pH values and high LBG concentrations. Oil-in-water emulsions containing 0.6% LBG at pH 5.0 exhibited the greatest consistency index and shear thinning behavior. No rheological tests were carried out on emulsion made at this pH with a lower concentration of locust bean gum Strong shear thinning behavior in colloidal systems is often a sign of breakdown of structure (Hunter, 1987). As large aggregates increasingly break up under the influence of the shear forces, the apparent viscosity begins to drop (Barnes, 2000). In contrast well dispersed emulsions at oil volume fractions below those that cause severe distortion of the shape of oil droplets, are known to exhibit Newtonian type behavior with no real sign of shear thinning. This result for CAS stabilized emulsion, at sufficient amount of sodium caseinate for the full coverage of the droplets, but without excess protein in the bulk, was for example confirmed by Dickinson and Golding (1997) and more recently by Day, Xu, Hoobin, Burgar & Augustin (2007). In both sets of studies, the excess amount of CAS in the solution lead to considerable shear thinning and was attributed by the authors to the presence of depletion flocculation induced by free protein. In much the similar way, our emulsions here exhibit only modest shear thinning and a behavior closer to Newtonian at pH values of 6 and 6.5, in absence of LBG. The electrostatic repulsion between the CAS covered droplets is sufficient to keep the droplets well dispersed. With locust bean gum added, the emulsions become strongly shear thinning, as is demonstrated by the graphs in Fig. 9 and 10. Some of the exhibited shear thinning can undoubtedly be attributed directly to LBG itself. Solutions of this gum in particular, tend to start shear thinning at relatively small shear rates. However, Wu et al (2009) have found that a pure solution of LBG at 0.5% has an almost Newtonian plateau between shear rates of 0.1 to 10 s^{-1} with an apparent viscosity of 0.2 Pas, nearly two orders of magnitude lower than our emulsion systems (with a comparable LBG content of 0.6% and pH=6.5). Thus, the extend of shear thinning seen in our emulsions is such that an additional factor must also play a significant role in

the observed shear thinning behavior of these fluids. Micrographs in Fig. 11 and particle size distributions of figure 3 both highlight the presence of emulsion droplet aggregates. Thus, it would be natural to attribute the additional factor in shear thinning of our LBG containing emulsions to the breakdown of aggregate structure under shear. Furthermore, since the strong pseudo plastic behavior of the emulsions occurs immediately and at the lowest shear rates studies 0.1 s^{-1}, with no sign of an initial low shear Newtonian plateau (Fig. 9 and 10), the aggregates must be rather fragile. This in turn points to the weak nature of attractive forces that must be holding the droplets together in these aggregates. Once again, while it is not possible to rule out other alternatives without carrying out much more elaborate experiments, the most obvious candidate for such weak forces in our systems seems to be the depletion interactions mediated by LBG.

Finally we mention that viscosity of the emulsion is hardly affected by coalescence as opposed to aggregation of the droplets, much as has been suggested by Surh (2006).

5. Conclusion

The aim of this study was to specifically investigate the influence of pH and locust bean gum concentration on the stability of CAS-stabilized emulsions during the storage at room temperature, freeze-thawing, and freeze-drying processes. The stability of all emulsions was highly influenced by the pH value, consequently most CAS-stabilized emulsions at pH 5.0, i.e., close to the isoelectric point of adsorbed proteins, were more susceptible to coalescence than those at higher pH values. However, the presence of LBG as a thickening agent considerably improved the stability against coalescence of most emulsions even at low pH values. However, we do find evidence that LBG causes flocculation of the emulsion droplets. We have argued that this is likely to be due to depletion flocculation induced by locust bean gum present in the bulk solution. In absence of LBG at higher pH values, away from the pI of the sodium caseinate, the flow behavior of the emulsions was closer to a Newtonian fluid. For example for an emulsion with 0.0% LBG at pH=6.5 the flow behavior index ~ 0.87. Evidence for the presence of flocs was also seen in the micrographs of emulsions with lower concentrations of LBG (0.2%), but once again were absent without LBG.

Emulsions are largely unstable when they are frozen and then break down after thawing particularly when they are freeze dried. Principally, non-reducing carbohydrates, e.g., sucrose, are used to improve the stability of emulsions to freeze-thawing and freeze-drying processes. For systems with sufficient concentration of locust bean gum there was some improvement in the stability of emulsions against coalescence following reconstitution of freeze dried sample. But particle size measurements revealed the presence of large particles in these reconstituted emulsions, both with and without LBG. This may well once again be flocs rather than coalesced droplets. The nature of these remains an interesting problem for an investigation in future.

This preliminary study provides insight into the stabilizing effects of locust bean gum on the CAS-stabilized emulsions at various pH values which can be also beneficial for both frozen low-calorie and freeze-dried emulsions. Nevertheless, the destabilization of emulsion droplets is highly complicated and further studies, e.g., electron microscopy is needed to gain further understanding of CAS-coated oil droplet interactions.

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