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Application of lysozyme in formation of multilayer emulsion containing caseinate

Master of Food Technology

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Contents

1. Abstract

The properties of oil-in-water emulsions stabilized by caseinate-lysozyme complexes were investigated. Complexes were prepared by the mixture of 0.8 wt.% sodium caseinate with various amounts of lysozyme (0-0.5% W/W) at neutral pH. The emulsions formed by the serum solutions were not stable at low lysozyme concentrations (0-0.2 % W/W), but were stable at high concentrations (0.3-0.5 % W/W). The effects of lysozyme on caseinate-stabilized O/W emulsions were studied. Multilayer emulsions (containing 0.4 wt.% caseinate and 0-0.5 wt.% lysozyme) were created by mixing a primary caseinate stabilized emulsion with lysozyme solutions. The emulsions were evaluated at pH 3.3 and 6.8. At neutral pH, the emulsions were stable in the initial presence of 0.1 wt.% lysozyme due to bridging flocculation, but unstable at high lysozyme concentration of 0.1-0.5 wt.% . In acidified emulsions, lysozyme had no effect on caseinate-stabilized emulsions. Therefore, at neutral pH, complexes formed with high caseinate-lysozyme ratio could not create stable emulsions. On the other hand, caseinate-stabilized emulsions could only stay stable when the lysozyme to caseinate weight ratio was 1:2.

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3. Introduction

Emulsion systems are of high importance in food industry. Many food products like milk, desserts, dressings, butter, mayonnaise, and ice cream fall into this category. Traditionally, the production of emulsions involves homogenization of two immiscible liquids in the presence of one or a combination of emulsifiers. The stability of emulsions relies heavily on the emulsifiers applied, upon whose abilities to form and maintain a protective layer around droplets against various environmental stress factors like pH, ionic strength, and thermal treatment. However, the emulsions produced with conventional emulsifiers and methods have limited stability against these stresses. These limitations are the main drivers for developing new strategies to improve qualities of emulsions. Multilayer emulsions have gained increasing interest as a potential to alter interfacial layer properties such as chemical compositions, electrostatic charges, thus the emulsions responsiveness against environmental changes. There are two ways to produce multilayer emulsions: 1) emulsions stabilized by complexes formed through two oppositely charged polyelectrolytes before homogenization; 2) electrostatic complexes formed at the interface after primary emulsions stabilized by an ionic emulsifier (layer-by-layer methods).

In the present study, the behaviours of lysozyme and caseinate in emulsion systems, and their potential for production of a multilayer emulsion system were evaluated. According to theory [1], the following hypotheses were made:

1) The use of a complex will render the emulsion less stable than the use of caseinate alone;
2) When a Layer by Layer (LBL) technique is used to form an emulsion at neutral pH, lysozyme induces destabilization of caseinate-stabilized emulsions at low lysozyme concentration, while stable emulsions are generated at high lysozyme concentrations.

4. Literature review

4.1. Lysozyme

Lysozyme is widely used in food as a preservative because of it enzymatic activity [2]. However, its protein properties are the subject of this research. Lysozyme is a small globular protein containing 129 amino acids, with a molecular weight of 14 kDa [3-5]. The primary tertiary structures of Lysozyme are given in Fig. 1 and Fig 2 respectively.

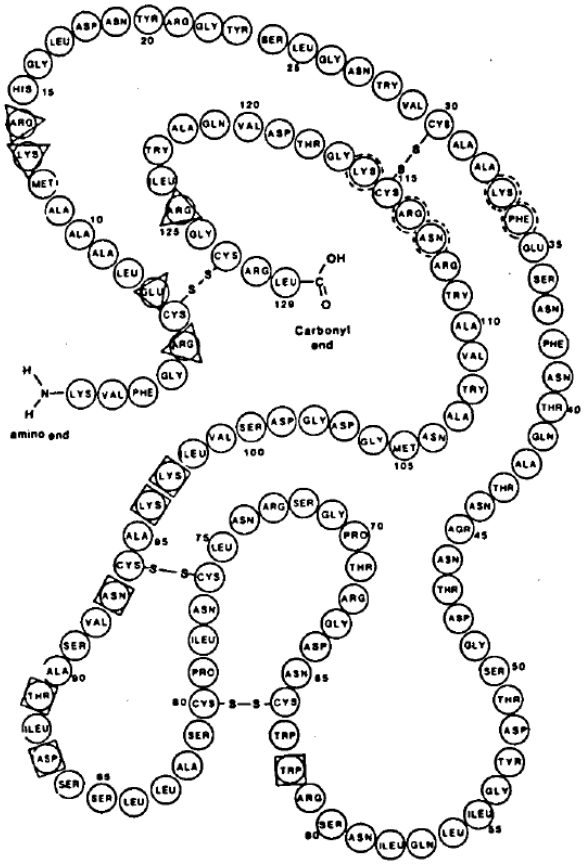

Figure 1. Lysozyme primary structure

Lysozyme is considered an amphiphilic protein. In solution there are 61 polar and 11 non-polar residues in contact with water molecules [6]. Lysozyme has a highly charged surface, when solution pH= 7.0, there are 8 exposed positively charged residues on the surface of lysozyme [7]. These unique characteristics give it a high isoelectric point pH of about 11 (11.35 for Chicken-lysozyme), and the ability to non-covalently interact with negatively charged polymers. Its molecule contains two domains: a highly

hydrophobic α-domain (46% of total residues), and a β-domain (20% of total residues, residues 36-84) [8]. With a large amount of closely packed hydrophobic residues presence in the core and surface of α-domain, the protein possesses a highly hydrophobic interior [9, 10]. The outer layer hydrophobicity of this protein is also considered very high. When lysozyme was adsorbed onto air-aqueous interfaces, 30-50% of the molecule protruded into the air, while almost all of BSA (Bovine serum albumin) molecule was buried in the aqueous phase [11, 12].

Figure 2. Lysozyme tertiary molecular structure [12]

Although lysozyme has a higher surface hydrophobicity than other globular proteins, it is not an excellent emulsifier when used alone. In a research analysing globular adsorption on oil-water interfaces, lysozyme showed a significantly less conformational change (4-7% reduction of α-helix) compared to bovine serum albumin (12-21% reduction of α-helix) which contains 13 more intramolecular disulphide bonds than lysozyme. This results from widely dispersed 4 disulphide bonds along the protein structure, plus the low flexibility of residues between these disulphide bonds. Consequently, lysozyme protein has a very strong pH and thermal conformational stability. Therefore, a longer adsorption and exposure time of the interior hydrophobic residues are needed in order to unfold at an interface. This feature is adverse to the formation of protein layer at the interface [5, 13].

4.2. Casein

4.2.1. Introduction

Naturally, caseins serve as a medium providing calcium and phosphate nutrition without causing calcification or amyloidosis of mother's mammary tissue [14]. In their natural states, they are secreted as 50-250 nm diameter casein micelles, with a colloidal calcium phosphate bridging α- and β-casein core and a κ-casein sterically stabilizing hairy out layer [15]. In bovine milk, the concentration of casein proteins is around 25 g/L, which consists of four different species: α_{s1}-, α_{s2}-, β- and κ-casein, with quantities (g/L) of 9, 4, 9, 3 [16-19].

4.2.2. Caseins structure

Despite a well-defined primary structure, its secondary and tertiary structures are not universally agreed. Because of the high content of proline residues, which prevent the formation of secondary structure [20, 21], caseins are expected to have little secondary structures (α-helix and β-sheet structures). Researches examining casein structure using FTIR (Fourier transform infrared spectroscopy) [22] and CD (circular-dichroism spectroscopy) [23] support this theory.

The lack of secondary structure makes caseins very flexible. A study [24] showed there was no energy difference between protein states before and after thermal treatment while it usually leads to globular protein "denaturation". Therefore, it has been deduced that there was zero or very little energy gap between the pre-heated and post-heated casein protein states. Therefore, in pure solution, caseins can exist in many different tertiary structures under the same environmental conditions in contrast to rigid globular proteins. Their transient structures have been supported by research using CD and NMR (nuclear magnetic resonance spectroscopy) [25-28].

4.2.3. Casein interaction characteristics

Some of the seryl residues in caseins are phosphorylated, which gives them a negative charge under neutral pH [29]. The protein charge is especially strong for α-/β- casein, this is not only because of their large amount of SerP residues, but also due to these residues being grouped in patches while those of others are spread randomly among protein molecules. These charged residues allow them to interact with oppositely charged particles such as ions (Ca^{2+}) and polymers [30].

The major casein proteins (α_{s1}- and β- casein) contain no cysteine residues, thus no inter- or intramolecular disulphide bonds are expected from them. On the other hand, the minor proteins, α_{s2}- and κ-casein, have two cysteine residues respectively. In the natural state, κ-casein can form polymers through intermolecular disulphide bonds with other κ-caseins forming aggregates of various sizes, and with other dairy proteins, most commonly, β-LG. On the other hand, α_{s2}-casein can only form monomer or dimer aggregates with other α_{s2}-caseins. However, there are no natural disulphide linkage between those two species of caseins [31, 32].

Table 1. Casein properties

	Content (%)	MW (kDa)	SerP No.	Cys No.	Charge at pH= 6.6
αs1-casein	40-45	23	8-9	0	-22
αs2-casein	10	25	10-13	2	-14
β-casein	35-40	24	5	0	-15
κ-casein	9-15	19	1-2	2	-3

All caseins contain hydrophobic and hydrophilic residues, making them amphiphilic. The spread of these residues on the structure are grouped into patches. This is especially true for κ- and β-casein. Almost all hydrophilic and charged residues are contained in the first 40 residues from the N-terminal region of β-casein, the others are highly

hydrophobic. In κ-casein the residues near the C-terminal region (residues 106-169) are polar, while the rest are mainly nonpolar. In αs1-casein, for example, residues 40-120 are strongly hydrophilic while rest are relatively hydrophobic. This amphiphilic nature of the primary structure can be translated into casein protein amphiphilic character because of its lack of secondary and tertiary structure [24, 33].

4.3. Caseinate

Although caseins are present as casein micelles under natural conditions, monovalent caseinate ingredients are a more popular form when interfacial functional characteristics are desired in industry. The production of caseinate involves the following processes: Acidification of milk to around its isoelectric point (pI=4.6) dissolves calcium phosphate and precipitates the casein proteins, then a monovalent alkali is mixed with the casein precipitates to neutralize and re-dissolve them, producing sodium or potassium caseinate [34]. Sodium caseinate is a widely used food ingredient, mostly as an emulsifier due to its amphiphilic and flexible characteristics. A sodium caseinate solution consists of a mixture of casein protein (α_{s1}-, α_{s2}-, β- and κ-casein) monomers and submicelles of their aggregation [35, 36].

Caseinate usually exist as individuals and small aggregates in solution when electrostatic repulsion is strong, and become aggregated when repulsion is screened. The highly hydrophobic group tends to attach to the nonpolar patches of other casein molecules. Whereas the negatively charge phosphoserines provide electrostatic repulsion between proteins, preventing them from forming larger aggregates with each other. The combination of both affects the size of casein aggregates, which are determined by solution properties (i.e. pH, ionic strength (pI), temperature). Increasing ionic strength by adding calcium salts will result in larger aggregation caused by bridging [37, 38]. Monovalent ions screen repulsive forces and allow the formation of small micellar aggregates. Additionally, lowering the solution pH close to casein pI will

also decrease the surface charge, therefore promote protein aggregation [39].

4.4. Emulsion

4.4.1. Introduction

Simple emulsions are a system that consist of incompatible liquids, where one liquid (the dispersed phase) is dispersed in another one (the continuous phase) as droplets. There are two common types of food emulsions: oil-in-water emulsions (O/W), like milk, mayonnaise, and vinaigrette; and water-in-oil emulsions (W/O) such as butter and margarine. These emulsions can be classified according to the size of dispersed droplets: macroemulsions have a droplet size range of 0.1-5 μm, nanoemulsions have a droplet size scale of 20-100 nm, microemulsions typically consist of droplet sizes of 5-20 nm. Of these emulsion classes microemulsions are usually thermodynamically stable while the other two are kinetically stable [40].

In addition to the emulsion categories discussed there are also some other emulsions that are commercially of less importance in the food industry: Oil-in-oil (O/O) emulsions can be formed by mixing non-polar and polar oil [41]; Water-in-water (W/W) emulsions can be distinguished as a system made of two immiscible biopolymer (polysaccharides and protein/polysaccharides) solutions [42, 43]; and multiple emulsions which consist of three phases, like oil-in-water-in-oil emulsions (O/W/O), and water-in-oil-in-water emulsions (W/O/W) [44]. Out of the wide range of emulsions, the simple O/W emulsion is the main subject of this research.

Emulsion systems are kinetically stable. As the number of dispersed droplets increases, the contact area between two phases increase, the direct contact between nonpolar and polar molecules is not favourable due to intermolecular interactions. Consequently, surface tension of the interface will rise, increasing the free energy in the system. While systems, according to thermodynamic dictum, tend to stay at their minimum energy states, a total phase separation will eventually occur where contact area is at a minimum

[45]. A third component can be used to decrease this unfavourable contact while maintaining the same area: namely emulsifiers. Proteins, due to their amphiphilic characteristics, are widely used as emulsifiers in O/W emulsions. As a consequence of hydrogen bonding, ionic interactions, Van der Waals forces, the hydrophobic and hydrophilic residues spread over the protein molecules will form segregated patches in their tertiary structure [46]. As the nonpolar head dissolves into oil phase, the hydrophilic patches will stretch into aqueous phase. This layer formed at the interface will decrease interfacial tension and provide steric repulsion and electrostatic repulsion between droplet interfaces, so the system can maintain stability over a longer period of time [47].

4.4.2. Emulsion properties

One of the most important aspects of emulsions is its ability to remain kinetically stable. There are several measurable properties that can be used to evaluate emulsion stability: these include droplet size distribution, compositions of the interfacial layer around the droplets and the continuous phase [48]. Fundamentally, it is the extent and rate of reaction between molecules in these phases, which translates as interactions between droplets that define the system they compose.

The interaction potential between droplets determines whether droplets will aggregate or stay as individuals. There are several interactions that are involved in determining the total interaction between droplets in the system: Van der Waals interactions, electrostatic interaction, steric interaction, depletion interaction, hydrophobic interaction, hydration interaction and thermal fluctuation need to be taken into considerations [49-51].

Emulsion droplet size and size distribution are essential aspects to characterize emulsions as they indicate interfacial contact area between oil and water phases [52]. They also play a vital role when it comes to determining chemical reactivity, stability,

rheology, and physiological efficiency of emulsions [48, 53]. One could behave significantly differently in terms of these characteristics from another emulsion if either droplet size or its distribution varies. A smaller droplet size usually means a lower frictional force according to the stroke's law [54]. This force contributes to the movement to the oil droplets, resulting in coalescence and flocculation. Therefore, emulsions with smaller droplet size generally stay stable for a longer time period than emulsions with larger droplets.

Interfacial protein load can also be used to assess emulsions. Firstly, higher surface protein coverage typically reduces direct contact area of polar and non-polar molecules, bringing interfacial tension to a lower level. Secondly, a more elastic interfacial film with higher hydrodynamic thickness can also be detected when there are more proteins present at the interface, which increases the steric repulsion between droplets as well as resistance to collapse of the interfacial layer. These all contribute to higher emulsion stability. On the other hand, when an insufficient amount of protein is present, one protein molecule could adsorb onto interfaces of two droplets, thereby resulting in bridging flocculation, which decreases emulsion stability significantly [55].

Zeta-potential is a parameter evaluating the electro-kinetic potential difference between aqueous phase and stationary layer attached onto droplet surfaces. This value indicates how strong the electrostatic repulsion between droplets is. As electrostatic interaction is one of the most important repulsion mechanism stabilizing emulsions, evaluating the electro-kinetic potential plays a significant role in determining emulsion stability [56].

4.4.3. Emulsifier

4.4.3.1. Introduction

Emulsifiers are vitally important for the emulsion system to stay stable. Water molecules can form hydrogen bonds with other water molecules, while the interaction between water molecules and oil molecules is van der Waals interaction because of the

absence of polar groups in the latter, which is relatively weak. Thus, increasing water and oil molecules contact would be unfavourable as it means replacing a stronger water-water interaction with a weaker oil-water interaction. In this case, the system will eventually collapse. To prevent this from happening, free energy needs to be provided, to decrease the unfavourable contact. The amount of free energy required can be described as [57]:

$$\Delta G = \gamma_i \Delta A$$

Where ΔG is the free energy required to maintain a contact interface between two phases, ΔA is the contact area, γ_i is the interfacial tension between two phases.

To maintain a relatively stable contact area between polar and nonpolar areas, another species of molecules need to be present to reduce the free energy required in oil and water system, namely emulsifiers. Under most cases, there will be nonpolar and polar groups present in emulsifier, when it accumulates at the interface of oil and water phases, their replacement of unfavourable direct contact between oil and water molecules with more favourable contact, nonpolar segments interact with the oil phase, and polar segments interact with the water molecules. The interfacial tension of system will decrease compared to the one without emulsifier, therefore the energy required to maintain a stable state will also decrease.

4.4.3.2. Emulsifier adsorption process

Once the interface between oil and water is formed through the emulsification process, proteins, because of its amphiphilic characteristics, tend to accumulate at the interface [46]. The nonpolar and polar residues will extrude into the aqueous and oil phases respectively. As a result, the layer formed can decrease the interfacial tension and prevent droplets coalescence. The entire interfacial layer formation process during homogenization contains three main steps:

1) Proteins spontaneously accumulate at the interface through convection [58, 59] ;
2) Protein molecules anchored onto interface through hydrophobic patches;
3) Protein conformation changes, exposing more hydrophobic groups and forms a cohesive film at the interface.

Only a fraction of the emulsifiers present will be adsorbed at the interface, as there are barriers to be overcome during emulsifier adsorption [50]. Firstly, when there is already adsorbed emulsifier at the interface, subsequent adsorption of aqueous emulsifier could be hindered due to the repulsive forces (e.g. electrostatic, steric). Secondly, the orientation of emulsifiers when they encounter the interface matters. For some globular proteins, it is important that hydrophobic segments contact with oil phase first to enable adsorption. Thirdly, formation of surfactant micelles in solution could have adverse effects on adsorption. Micelles, in contrast to the amphiphilic characteristics of surfactant, tend to have a hydrophilic out layer, which prevents the surface activity of the surfactant as individual molecule.

4.4.3.3. Emulsifier effectiveness

Emulsifier effectiveness is defined as the ability to stabilize certain areas of the interface, and its ability to maintain this stability. There are usually three indexes used to describe effectiveness: emulsifying capacity (EC), emulsifying activity (EA) and emulsion stability index (ESI). EC is the evaluated as the amount of oil [60] stabilized by 1 g of proteins; EA is described as the maximal contact area (cm^2) that can be stabilized by 1 g of protein; ESI is the ability of protein stabilized emulsions to stay stable [61]. While emulsion stability is highly related to the environmental properties like pH, ionic strength and oil type etc., properties of emulsifier molecules fundamentally determine its behaviours during adsorption and post-adsorption processes, thus the essential properties of the formed interface.

Protein surface activity is essential in determining protein adsorption speed. It is highly

related to the physicochemical properties of proteins, such as conformational flexibility, hydrophobicity and hydrophilicity distribution, surface hydrophobicity.

The protein surface activity determines the migration, adsorption, unfolding and cohesion abilities of proteins [61-65]. There are two aspects that determine the rate of the migration step: the size of the binding protein or its aggregates as emulsifiers with smaller size typically move faster in aqueous phase and will reach the interface prior to the larger ones; and the availability of the adsorption sites of emulsifiers, which are usually hydrophobic groups [61, 66]. When a protein first reaches the interface, it is important for adsorption that hydrophobic residues attach and anchor the molecule onto the interface swiftly. Thus accessible nonpolar residues play an important role in the first stage of adsorption [67]. Generally 57% of the accessible residues need to be nonpolar on the protein's surface for globular proteins be absorbable, while the balance between charged and non-charged polar residues does not matter [68]. A group of more patched hydrophobic residues in protein would result in a shorter adsorption time than when hydrophobic residues exist as more isolated individuals.

Additionally, the flexibility of proteins in the solution determines the total interface area that can be stabilized, and thus oil droplet size. Ideally, more contact area of two phases shall be covered by sufficient hydrophilic and hydrophobic materials. A more flexible surfactant can extend more at the interface and cover a larger area, thus allowing production of smaller droplets [69]. Casein, as a flexible protein, only requires a minimum protein coverage of 1 mg/m2 to generate stable emulsions, whereas that of more rigid whey proteins requires 1.5 mg/m^2 [70, 71]. Additionally, more flexible proteins expose hydrophobic residues faster after being adsorbed onto the interface.

The magnitude and sign of charged residues on the surfactant molecule determine the charge of droplet it covers, as the covered interface will show similar electrical characteristics with the surfactant [72]. Surfactants with a higher net charge will usually contribute to a higher charged interface, providing a stronger electrostatic repulsion between emulsions droplets, therefore a more stable emulsion compared to the

emulsion system stabilized by surfactants with weaker charge.

4.4.3.4. Effects of environment on emulsions

The environment affects emulsions in various aspects, like phases with higher viscosity can decrease the movement rate of droplets, and result in an extended stability period. Additionally, the interfacial film formation is governed by emulsifier-emulsifier, emulsifier-interface, and emulsifier-phases interaction, which depend on environmental conditions such as protein concentration, solution pH, temperature, ionic strength and homogenization process, which will be discussed in detail.

Increasing ionic strength of the protein solution will screen protein residue charge, impairing protein electrostatic interaction between droplets, which is referred as electrostatic screening. Therefore, before emulsification, emulsifier aggregate states are highly affected by solution ionic strength, which will eventually affect droplet diameter and protein coverage of the emulsion produced. Generally, adsorbing emulsifier with a smaller size can generate emulsions with smaller droplets because of their faster diffusion time [73], while more aggregated proteins will be adsorbed as aggregates, resulting in a much higher protein coverage. Mulvihill [74] found aggregated caseins have lower emulsifying capacity and surface activity than dispersed caseinate, casein micelle stabilized emulsions have a higher droplet size and more surface protein coverage results from casein micelles that are adsorbed at the interface as a whole, which increases the thickness of interfacial film, generating a more stable emulsion. A more detailed research [75] about emulsions stabilized by high-calcium aggregated (Ca^{2+} concentration>0.08%) casein solution showed similar results. Increasing calcium chloride concentration in protein solution from 0.08% to 0.2% boosted protein coverage and turbidity of the emulsions, whose droplet size increased by about 0.25 μm. However, increasing the caseinate concentration alone, which is known to induce formation of casein polymers, only increases the protein coverage while droplet size of the emulsion

remains unchanged [70, 71, 75]. Another study manipulating casein aggregation states in solution by demineralizing a casein micelle solution also showed the same results [76].

For the same reason, increasing ionic strength contributes to instability after emulsions are made. The effects of monovalent particles can be described as screening droplets charges and thus weakening electrostatic repulsion. On the other hand, for multivalent particles, the screening effect is much stronger. With an additional effect called bridging flocculation, where one particle associates with two droplets [77].

The changes in the species proportion of the absorbed layer are also related to the ionic strength change as electrostatic repulsion between proteins plays a part in the initial stage of adsorption. Increasing ionic strength of the bulk casein solution results in a higher percentage of α_s-casein present on interface while that of β-casein decreases, while κ-casein attachment changes were not proportional to the Ca^{2+} concentration increase [75].

The environmental pH of the bulk solution plays a similar role in affecting protein surface residues ionization extent, which determines electrostatic interaction between proteins. Therefore it influences protein emulsifying properties by affecting its solubility, molecular conformation and surface properties [78]. A low emulsifying capacity and high protein load at the interface can be detected when solution pH is near to protein pI, where proteins form bigger aggregates in the solution. When pH moves away from pI, EC of proteins increases. Similarly, screening droplet surface charge by adjusting solution pH to pI of proteins after emulsions are formed could have adverse effects on the emulsion stability.

Protein concentration in the bulk solution also affects interfacial layer properties [61]. Lower protein concentration stabilized emulsions will usually have a thinner interfacial film. Under low concentrations, adsorbed β-casein molecules have fewer tail structures stretching into aqueous phase, as the entire protein backbone attaches to the interface,

whereas more tail structures and multilayer interfacial formation have been observed under high concentrations [46].

The dispersed phase volume fraction determines emulsion physiochemical properties in terms of droplet size and interfacial composition [79]. In most cases, it is well recognized that increasing the oil volume fraction in O/W emulsions increases droplet size [80, 81]. This is likely to be due to the amount of available emulsifier being insufficient to cover the newly generated oil surface, and the coalescence of these droplets [82, 83]. However, the effects of oil volume fraction on droplet size are emulsifier concentration dependent. Under emulsifier concentrations that are not sufficient to cover all the oil surface area, increasing oil volume fraction would result in a droplet size rise. Whereas when sufficient or slightly excessive emulsifier concentrations are present, higher oil volume fraction does not generate an emulsion with larger droplets [84, 85]. In emulsions with slightly excessive amount of emulsifier, increasing oil volume fraction in emulsions will result in a decline of emulsifier to oil surface ratio. This could result in an interfacial layer change formation from multilayer to thin monolayer, thus significantly decreasing saturated protein surface coverage [75, 86].

4.4.4. Caseinate, lysozyme as emulsifier

Caseinate is an excellent emulsifier and is used widely in food manufacture. With high conformational flexibility and spread of hydrophilic and hydrophobic patches, casein proteins have a strong tendency to adsorb onto water-oil interfaces, at a relatively fast speed. After being adsorbed, caseins can stabilize emulsion in multiple ways other than lowering surface tension. Their flexibility allows part of casein molecules to protrude very far into the aqueous solution and thus prevent droplet contact sterically.

Additionally, the charged residues of interfacial casein can provide strong electrostatic repulsion between droplets. The electrostatic repulsion between the proteins on the

same surface can also facilitate the protrusion of the some of the hydrophilic portions of the proteins into aqueous phase which increases stability through generating steric repulsion [87].

As for single type casein stabilized emulsions, β-casein forms the thickest adsorbed films [88]. According to literature on β-casein adsorption behaviour on a hydrophobic surface, the adsorbed β-casein structure is well defined. Hydrophobic residues (160-170) compose of trains attaching to the interface, while residues 40-50 stretch into the aqueous phase [89, 90] (Fig. 3a).

The highest charged casein, α_{s1}-casein, forms a highly charged layer when adsorbed at the interface [91]. But due to the distribution of hydrophobic and hydrophilic residues among the structure, it forms a loop conformation (Fig. 3b), resulting in a thinner adsorbed interfacial layer [89].

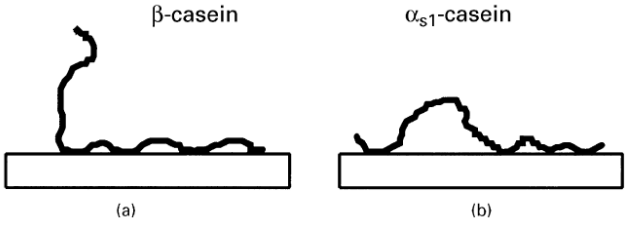

Figure 3. α- and β-casein conformation at O/W interface

The stabilization mechanism of α_{s1}- and β-casein differs, while the former mainly stabilizes droplets by electrostatic repulsion, β-casein also provides steric repulsion. Emulsions containing α_{s1}-casein destabilize when solution pH is adjusted close to pI or ionic strength is high. While the repulsion of β-casein stabilized emulsions tends to stay positive regardless of pH and ionic strength change, indicating β-casein stabilized emulsions tend to be stable against a wider range of pH and ionic strength environment than α_{s1}-casein emulsions [92-94].

Generally, β-caseins are more surface active than other caseins, and more are present at the interface at caseinate concentrations lower than 2%. However, a less surface-active

casein, α_{s1}-casein will be adsorbed in preference to β-casein when the caseinate concentration exceeds 2%, while κ-casein adsorbed the least regardless of concentration [95, 96].

On the other hand, increasing caseinate content to above 3% will decrease emulsion creaming stability significantly. This is because of special self-assembly characteristics of unabsorbed caseins in the aqueous phase, which will induce depletion flocculation due to osmotic effects [97, 98]. This effect can be hindered by introducing a small amount of calcium/sodium ions, resulting in an increase in size of unabsorbed aggregates that are not able to induce depletion flocculation [99-101].

Ionic sensitive caseins like α_{s1}- and β-casein make up a large proportion of the interfacial protein content, thus caseinate stabilized emulsions share the similar vulnerability to, especially, calcium ions. These ions may bond with charged residues in proteins from two droplet surfaces, result in flocculation of droplets. On the other hand, bonding with the proteins on the same interface screens the charges and diminishes electrostatic repulsion, and the hydrodynamic thickness of adsorbed protein layer will decrease as a result [102]. Presence of monovalent ions like sodium can weaken the adverse effects of calcium on emulsion stability [101]. Lowering the pH near to the pI of caseins can also destabilize casein stabilized emulsions, as a consequence of lowering repulsive protein charges [103].

4.5. Multi-surfactant emulsions

4.5.1. Electrostatic complex

Under different physicochemical medium properties, two types of proteins can interact with each other in various ways (i.e. hydrophobic interactions, electrostatic interaction, van der Waals forces, polyelectrolyte bridging), usually forming rod-like, or spherical

aggregates [104]. Due to the more complexed protein structure, protein-protein electrostatic interaction will not neutralize all surface charge in contrast to protein-polyelectrolyte interaction [105]. The remaining charges of proteins are vital in determining formation of complexes. When solution pH is away from pI of proteins, with a low ionic strength, complexes formed tend to be more linear because of the high electrostatic repulsion generated by the remaining charges. On the other hand, when the solution has a high ionic strength and a pH near to pI, assemblies are more spherical [106].

Due to the highly charged protein surface of lysozyme and caseins, they also tend to associate with each other through electrostatic interaction, in a pH range between the pI of the two proteins [107]. Researchers [108, 109] investigating the association between different species of caseins and lysozyme found out that lysozyme has the strongest bound tendency with α_{s1}-casein, which has the most charges among all caseins, and second strongest with β-casein, whereas it only associates with a small amount of lower charged κ-casein. This also indicates that hydrophobic interaction plays an insignificant role [110].

As the electrostatic interaction involves protein charges, the solution ionic strength has a significant effect on association between lysozyme and caseins. It was suggested by an early study that formation of electrostatic complex between lysozyme and β-casein at 0.1 ionic strength is impossible, as charged residues are effectively screened [111]. It might be the case since the protein concentration used in that study was relatively low (1.53-4.7 μg/mL). However, some other studies applying higher protein concentrations show different association behaviours, that all lysozyme will associate with α_s-casein until NaCl concentration is greater than 4%; β-casein and whole caseinate association affinities decreased as ionic strength increased; there was no association between lysozyme and κ-casein at any ionic strength [107, 110].

Ionic particles screen charged residues, determining the number of accessible charge residue left for protein-protein interaction between lysozyme and caseins, thus it also

influences the association ratio of casein and lysozyme. The interaction between β-casein and lysozyme has been thoroughly researched. In a 0.1% β-casein system, every lysozyme has been observed to associate with three β-casein molecules when the solution ionic strength is 0, and increasing the solution ionic strength to 1.0 M resulted in an association ratio of 2:3 [112]. In other research where lysozyme was added into whole caseinate with 50 mM NaCl and 50 mM K-phosphate buffer present, one mole of αs1-casein associated with more than one mole of lysozyme [110].

The structure of complexes was also examined under conditions where the weight of lysozyme was 0.1 to 0.5 times that of caseinate. Under these conditions, the complexes formed tended to show a rod-like structure (Fig. 4) [113]. Another research brought out a different model for the complex, which can be described as a lysozyme stabilized micellar casein. The difference between the two studies may be due to the complexes in the latter study being formed under a pH of 5.0/10.0, which is near the pI of casein/lysozyme [114]. At the pI proteins have less charge and thus less electrostatic repulsion in the complex and so the dominating interaction in the complex formation was hydrophobic interactions. Consequently, a more aggregated complex can be formed, namely micellar structure.

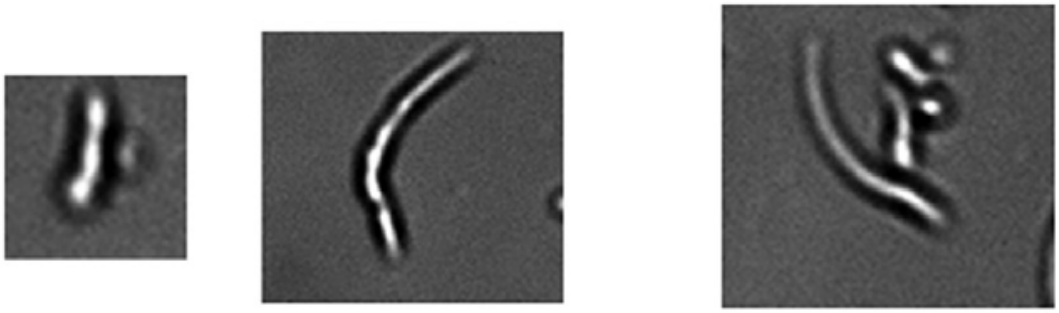

Figure 4. Casein and lysozyme aggregation states at different lysozyme to casein W/W conditions ionic strength= 10 mM (0.12; 0.16; 0.41) [113]

4.6. Competitive adsorption between lysozyme and caseins

Under most cases where globular proteins are involved, protein composition on the interface is determined primarily by the protein attachment speed, the ones arriving and adsorbed onto the surface earlier will stay on the interface [115]. Although there is no direct research on the competitive adsorption behaviour between lysozyme and caseins on the oil-water interface, studies done in air/water systems showed the similar results that no replacement occurred between lysozyme and caseins [111, 116].

4.7. Complexes as emulsifier

The application of the electrostatic complexes formed with proteins and polysaccharides through as emulsifiers draws huge interest. There are two ways of applying oppositely charged emulsifiers. Firstly, if complexes are formed prior to emulsification, this emulsifier has better emulsifying ability, and can generate smaller droplets compared to the emulsions stabilized by one of the surfactants alone [117]. The second method is to form an emulsion using protein alone, and then to add polysaccharides. This will make a multilayer emulsion with different interfacial properties (e.g. stability against pH, ions) compared to single ones [118].

Electrostatic complexes have better emulsifying ability and better ionic stability compared to when used alone. Ye [119] used a mixture of equal weight of β-lactoglobulin and lactoferrin as emulsifiers and tested the properties of the emulsions formed. He found out that droplet size formed by complexes are smaller than that of β-lactoglobulin alone, but increasing protein (complexes) concentration had little effect on diminishing the size, especially when concentration exceeded 1%, where complexes and single protein generated similar size of droplets. The emulsions formed by complexes were stable despite the zeta-potential of the complexes stabilized emulsions was close to zero. Another study [120] showed that emulsions stabilized by assemblies of β-casein and lactoferrin had better stability against calcium ions compared to β-

casein stabilized emulsions.

4.8. Multilayer emulsions

4.8.1. Formation of multilayer emulsions

The characteristics of electrostatic interaction between charged droplets and oppositely charged particles can be applied to form a multilayer at the interface. This system can be formed by stabilizing emulsions with a single emulsifier to form a primary layer, and then adding oppositely charged particles into the system which will adsorb onto the primary layer to form a secondary layer. Fig. 5 shows the visualization of the general multilayer system formation process.

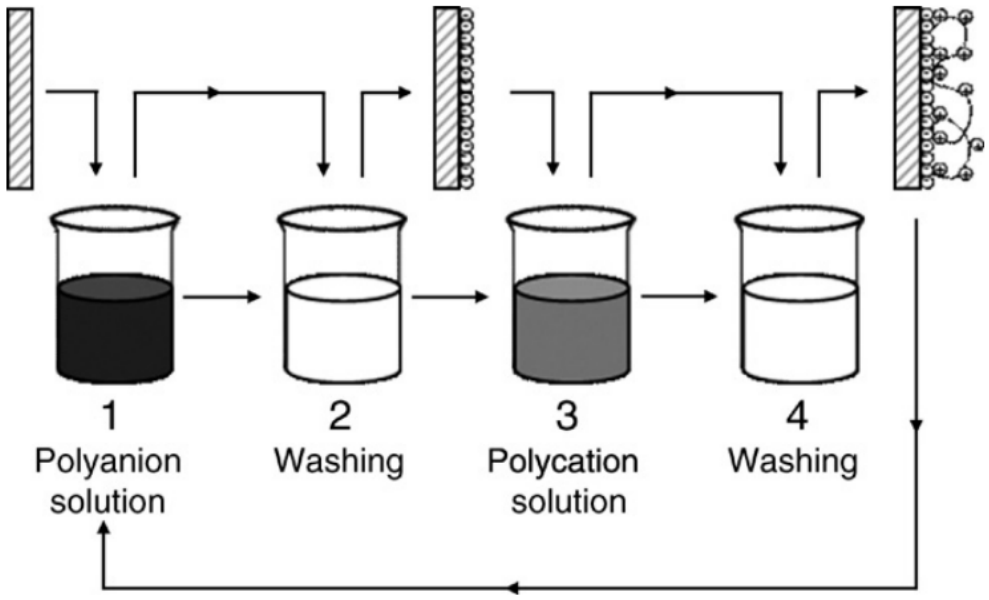

Figure 5. Multilayer system formation process [118]

To form multilayer effectively, it is important to 1) maximize the primary layer coverage; 2) minimize the excessive surfactant present in the aqueous phase. A saturated interface containing sufficient emulsifier is important because it minimizes the possibility of a second surfactant adsorbing directly at the interface. It is essential to decrease the aqueous emulsifier concentration to maintain emulsion stability, as the presence of aqueous emulsifier could form complexes with the opposite charged

surfactant as well, which might induce depletion flocculation[60].

There are three main methods can be used to produce achieve this aim. First one is the saturation method, where the emulsifier concentration added is in excess. Second way, centrifugation method, which eliminates the aqueous phase surfactant by separating aqueous and oil phases through centrifugation, this step is followed by then re-dispersing the oil layer into buffer solutions. Another method is to separate two phases by filtration, and then disperse the oil droplets into the buffer solution[60].

4.8.2. Factors affecting multilayer emulsions

As mentioned before, pH plays a significant role in determining protein charge. In multilayer emulsion systems, the pH of both surfactants is governed by environmental pH, it is thus necessary to choose a pH which gives them sufficient opposite charge to facilitate their electrostatic interaction [121]. In the case of two proteins, the solution pH should be between pI of two proteins.

The ionic strength of the solutions should be controlled as well. A higher environment ionic strength will result in a counter-ions layer around the primary interfacial layer, which decreases the electrostatic attraction between primary layer and newly added polyelectrolytes, which eventually will hinder the formation of a secondary layer. Increasing ionic strength to sufficiently high after the formation of a multilayer might detach the secondary layer as a result of decreasing electrostatic attraction between layers.

The concentrations of both polyelectrolytes present determine the behaviour of the emulsions. As for the surfactant forming the primary layer, the amount should be

sufficient to saturate the accessible interface between the two phases as mentioned before. As for the concentration of the secondary polyelectrolytes, it is important to maintain a level where there is a sufficient amount to prevent bridging flocculation while lower than the level where there might be depletion flocculation. A model containing colloidal particles and oppositely charged polymers can be applied to describe the relationship between concentration and emulsion behaviour [122]:

$$C_{sat} = \frac{3\emptyset\Gamma_{sat}}{r}$$

$$C_{ads} = \sqrt{\frac{60\emptyset\Gamma^2{}_{sat} r_{PE}}{r^3}}$$

$$C_{dep} = \frac{M}{N_A}\left(\frac{-1+\sqrt{1-8vX}}{4v}\right)$$

Where

$$X = \left(\frac{w_{dep}}{k_B T}\right)\frac{1}{{}_{crit}2\pi r_{PE}^2\left(r+\frac{2}{3}r_{PE}\right)}$$

where ϕ is the volume fraction of the dispersed particles, Γ_{sat} is the surface load of the polyelectrolyte saturation at primary layer (kg/m2), r is the radius of the spherical particles (m), assuming a monodisperse solution, rPE is the effective radius of the polyelectrolyte molecules in solution (m), M is the molecular weight of the polyelectrolyte (kg/mol), v (=4π $r^3{}_{PE}$ /3) is the effective molar volume of the polyelectrolyte in solution (m3), N_A is Avogadro's number, w_{dep} is the strength of depletion attraction at droplet contact, which depends on droplet size and non-adsorbing concentration of the polyelectrolyte, k_B is the Boltzmann's constant, and T is the absolute temperature.

The effects of secondary polymer concentrations on colloidal particle aggregation states are illustrated in Fig. 6. When secondary surfactant concentration is lower than C_{ads}

or C_{sat}, bridging flocculation might happen; the ideal concentration is between C_{ads} or C_{sat} and C_{dep}, where the amount of secondary polyelectrolyte is sufficient enough to saturate the interface, but without enough aqueous polyelectrolytes to promote depletion flocculation; when the concentration is higher than C_{dep}, the system tends to be unstable due to depletion flocculation.

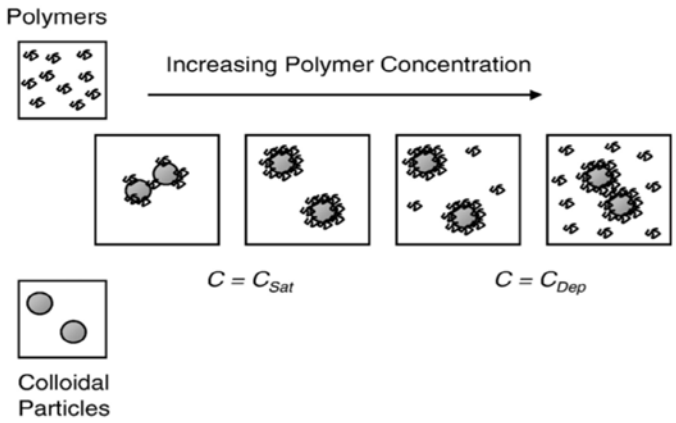

Figure 6. Schematic illustration of effects of polymer concentration on emulsions droplets

5. Preliminary experiments

5.1. Methods

5.1.1. Protein solutions preparation

Preparation of a caseinate 10 wt.% weight ratio solution:

The required amount of sodium caseinate Alanate 180 powder (Fonterra, New Zealand) was weighted. The powder was then added slowly into a glass beaker containing the desired amount of Milli-Q water of 50 ºC under stirring at 700 rpm. The mixture was stirred for 2 hours before being put into chiller for complete hydration and de-foaming overnight.

Preparation of lysozyme 5 wt.% solution:

The required amount of Egg white lysozyme E1105 (Delvozyme, France) was weighed

and added into a beaker containing the desired amount of Milli-Q water with room temperature. The mixture was stirred for 2 hours before being put into chiller for complete hydration and de-foaming overnight.

Preparation of mixed protein solutions:

12.8 g of 10 wt.% caseinate solution and lysozyme solution containing phosphate buffer were mixed under gentle stirring, an 80 g of final mixture containing 0.8 wt.% caseinate and 0.1/0.2/0.3/0.4/0.5 wt.% of lysozyme with 40 mM phosphate buffer (pH=6.4-6.7) was made. The mixture was stirred at 200 rpm for 2 hours.

5.1.2. Emulsion preparation

Casein-stabilized emulsion preparation:

The required amount of Canola oil (Sunnz, New Zealand), protein solutions and Milli-Q water were mixed to make a mixture containing 20 wt.% oil and 0.8/0.9/1.0/1.5 wt.% caseinate. The mixture was initially homogenized using a Bench homogenizer (Labserv) for 3 min to produce a coarse emulsion. Then the coarse emulsion was put through two-stage homogenizer (FBF ITALIA, Italy) with a first stage pressure of 70 MPa and a second stage pressure of 5 MPa. Two runs were conducted with 3 min pre-run for more uniform homogenization. Sodium azide (0.02 wt.% was then added to prevent microbe growth. Emulsions were left to sit for 90 min under room temperature before further procedures.

Mixed protein stabilized emulsion preparation:

Mixed protein solutions were mixed with 20 g of Canola oil (Sunnz, New Zealand),

generating a mixture with 20 wt.% oil, 0.64 wt.% caseinate, 0.08/0.16/0.24/0.32/0.4 wt.% lysozyme. The mixture was made into an emulsion through the procedure mentioned above, with only one modification of changing the first stage pressure from 70 MPa to 20 MPa.

Multilayer emulsion preparation:

50 mL of caseinate-stabilized emulsion was mixed with a 50 mL lysozyme solution containing phosphate buffer, giving a final mixture with 10 wt.% oil, ionic strength of 40 mM, 0.4 wt.% caseinate, and 0.1/0.2/0.3/0.4/0.5 wt.% lysozyme. The mixture was stirred at 200 rpm for 2 hours.

5.1.3. Particle size analysis

Light scattering technique is one of the most frequently used ways to measure particle size. The Mastersizer 2000S Hydro (Malvern, U.S.) was used to measure droplet size of the emulsions. Samples were diluted to 1:400 with 10 mM phosphate buffer (pH 7.0).

Emulsion droplet size was measured using same methods as Ye [123]. Measurement was performed at room temperature using Mastersizer 2000 (Malvern, U.K.). The following analysis parameters were used: material refractive index used was 1.47 for oil in water emulsions with an absorbance value of 0.001; RI of dispersant was 1.33 for water. The stirring speed was 3000 rpm.

Droplet size was expressed in two ways: Sauter-average diameter also called surface-weighted $d_{3,2}$ (μm) and De Brouckere diameter or volume-weighted $d_{4,3}$ (μm):

$$d_{3,2} = \sum \frac{n_i {d_i}^3}{n_i {d_i}^2}$$

$$d_{4,3} = \sum \frac{n_i {d_i}^4}{n_i {d_i}^3}$$

Where n_i is the number of emulsion droplets with diameter of d_i.

5.1.4. Phase protein content analysis of emulsions

Phase separation:

About 25 g of emulsions were weighed into a centrifuge tube and the exact weight was recorded. Centrifugation parameters applied were 45,000 g, 20 °C and 40 min. After centrifugation, the serum was removed carefully using syringe. The serum was then filtered using 0.45 μm and 0.22 μm filter paper in session.

Protein content analysis of phases:

The serum protein content was measured by Kjeldahl analysis. 25 mL of concentrated sulphuric acid and 2 pellets of potassium were added into Kjeldahl tubes containing weighted serum samples. Digestion was conducted under 420 ºC for at least 80 mins. Distillation was conducted using Kjeltec with 8 mL of concentrated sodium hydroxide and heated for 4 min, steam was collected using 15 mL 4 wt.% boric acid solution containing indicator. Titration was conducted using 0.1 M HCl.

The interfacial protein concentration weight by volume (C_{int} (% W/V)) is calculated by:

$$C_{int} = \frac{C_T \cdot M_T - C \cdot V}{V_{int}}$$

Where C_T is the protein concentration of the primary emulsion wt.% , M_T is the weight of the primary emulsion (g), C is the protein concentration of the serum layer (% W/V), V_{int} is the volume of the serum layer [60].

Surface protein load calculation:

The surface protein load Γ is calculated as:

$$\Gamma = \frac{C_{int} \cdot d_{32} \cdot \rho}{6M}$$

Where C_{int} is the protein concentration of serum layer (% W/V), d_{32} is the volume-srface mean droplet diameter (μm), M is the droplet volume fraction, ρ is canola oil density=0.906 g/mL.

5.2. Results

5.2.1. Protein solution interaction

Aggregation was spotted in the mixed protein solutions. The solution with 0.1 wt.% lysozyme had the highest turbidity, and the minimal amount of precipitate. As the lysozyme concentration in the system increased the turbidity of the solution decreased and the amount of precipitates increased.

5.2.2. Emulsion preparation

Preparation of emulsions with mixed protein solutions was unsuccessful. At lysozyme concentrations of 0.1-0.2 wt.% , when coarse emulsion was put through two-stage homogeniser and at about 1 min running time, aggregation appeared, which jammed and completely stopped the homogeniser. However, emulsions made with a mixed protein solution containing high lysozyme concentrations (0.3-0.5% W/W) were stable.

5.2.3. Protein content analysis of caseinate-stabilized emulsions

During the production of sodium caseinate stabilized emulsions, increasing the concentration of sodium caseinate in the system resulted in an increase in surface protein load, as well as a rise in serum protein concentration.

Fig.6 shows the protein surface load as a function of sodium caseinate concentration used in the emulsions. While the general rise in the surface protein load as more protein present used is consistent with the previous studies [75, 124], the actual surface protein loads in the present study are significantly lower than that in others (i.e. 0.29 μg/m2 compared with 1 μg/m2 at caseinate concentration of 1.5%).

Fig.7 shows the effects of Na-CN concentration on the protein concentrations of the emulsion serum phases. Higher protein contents in the serum layer can be observed in emulsions produced with higher initial caseinate concentration.

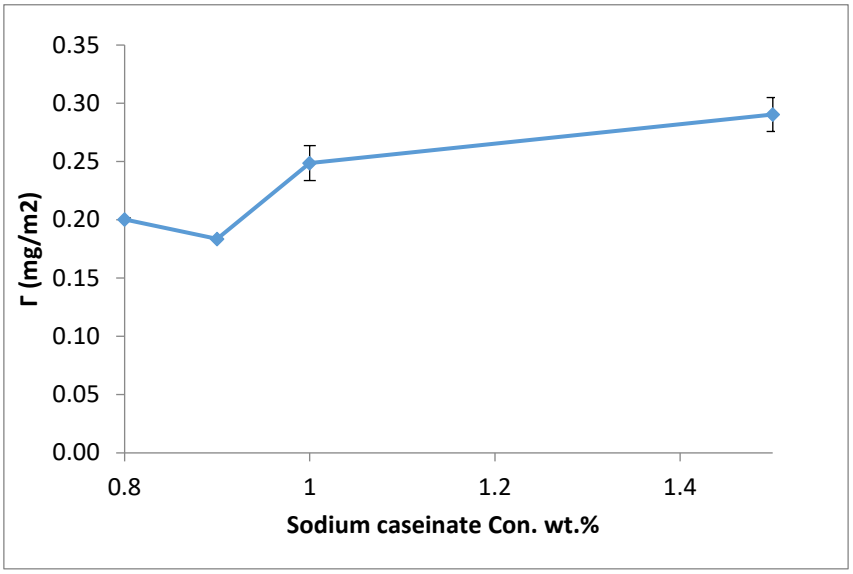

Figure 7. Effects of protein concentration on surface protein load of caseinate-stabilized emulsions (20 wt.% oil) 3 replicates

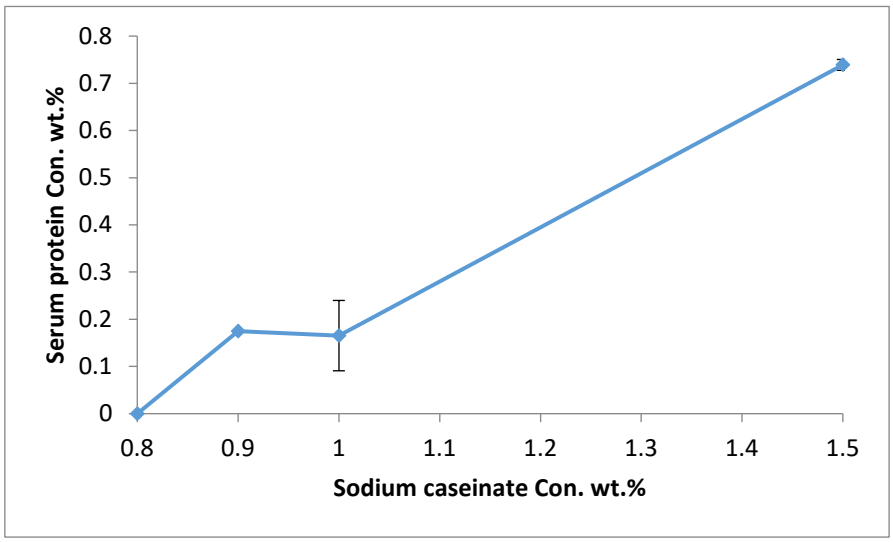

Figure 8. Effects of protein concentration on serum protein content of caseinate-stabilized emulsions (20 wt.% oil) 3 replicates

5.3. Discussion

Due to the high positive charge of lysozyme at neutral pH [7], its presence can induce aggregation of negatively charged caseins. As the concentration of lysozyme rises, the size of aggregates increases and eventually precipitation appears as a result of neutralization of casein charges, which is consistent with the previous study [113, 125].

Due to the limited analysis of both mixed protein solutions and the emulsions stabilized by complexes, no certain conclusions about the mechanism of emulsion aggregation can be drawn. Nevertheless, a likely hypothesis is that one protein complex contains multiple casein molecules, making many oil droplets attach to one compound, some of which associate with caseins from another complex. Consequently, a network structure can be formed. On the other hand, at high lysozyme concentrations, most complexes formed by lysozyme and caseinate precipitated, the serum contained either more single lysozyme molecules or complexes with different structure, i.e. a neutral interior with a surface consists of lysozyme molecules [113].

In the emulsions stabilized by caseinate, the interface protein load increases as increasing amounts of protein is used in the emulsion preparation, which in consistence with previous studies [102]. Again, the interfacial protein load in the present study is lower than that of the other researchers (e.g. 0.29 μg/m2 in the present study compared with 1 μg/m2 in the study of Srinivasan at caseinate concentration of 1.5%) [75, 124] . As suggested by the study of Srinivasan, increasing homogenization pressure results in a drop of interface protein coverage [75]. This lower protein surface load in the present research is attributed to the relatively high homogenization pressure applied (70 MPa) compared to that of Srinivasan (10 MPa).

6. Multilayer emulsion experiments

6.1. Methods

6.1.1. Solution preparation

Protein solution preparation procedures were the same as mentioned in the section 3.1.1.

6.1.2. Caseinate-stabilized emulsions 20 wt.% oil preparation

Emulsion preparation procedures were the same as mentioned in the section 3.1.2.

6.1.3. Preparation of multilayer emulsions

Lysozyme solution was mixed with Milli-Q water to make a 50 mL solutions with final lysozyme concentrations of 0.2/0.4/0.46/0.52/0.6/0.8/1 wt.% . The pH of the solutions

was adjusted to 2.0-2.1 using 1 M HCl solution (0.8-1.0 mL used). Then the pH adjusted lysozyme solutions were mixed with 50 mL of caseinate-stabilized emulsions. The mixture was stirred gently (100 rpm) for 5 min before its pH was adjusted to neutral (6.6-7.0) using NaOH (0.8-1.0 mL used). Then the mixture was kept under constant gentle stirring (200 rpm) for 1 hour for complete interaction.

Another group of mixed emulsions were prepared with the exact same procedure except for pH neutralization step, to enable the effects of lysozyme on caseinate emulsions under an acid pH environment to be examined.

6.1.4. Particle size analysis

Particle size analysis procedures were the same as mentioned in the section 3.1.3. Dilution was carried out with 10 mM phosphate buffer (pH 7.0) for neutral group or 10 mM di-sodium phosphate citric buffer (pH 2.6) for acid group.

6.1.5. Zeta-potential analysis

Zetasizer Nano-ZS (Malvern, U.K.) was used for sample zeta potential measurement. Dilution was carried out with 10 mM phosphate buffer (pH 7.0) for neutral group or 10 mM di-sodium phosphate citric buffer (pH 2.6) for acid group.

6.1.6. Protein content analysis of phases

Protein content analysis procedures were the same as mentioned in the section 3.1.5.

6.1.7. SDS-PAGE analysis of serum of acidified emulsions

Reagent:

Running buffer: 4 wt.% acrylamide, 125 mM Tris-HCl, 0.1 wt.% SDS, 0.1 wt.% TEMED, 0.1 wt.% APS

Sample buffer: 100 mM Tris-HCl, 4 wt.% SDS (sodium dodecyl sulphate), 0.2 wt.% Bromophenol blue, 20 wt.% glycerol, pH approx. 6.8.

Gel Staining: 3 wt.% Coomassie brilliant blue R, 20 wt.% isopropanol, 10 wt.% glacial acetic acid

Gel destaining: 10 wt.% isopropanol, 10 wt.% glacial acetic acid

12 wt.% Miniprotean TGX Precast gel was purchased from BioRad, U.S.

Procedures:

The serums of emulsions were diluted 1/2/4/4.6/5.2/6/8/10 times with Milli-Q water according to lysozyme addition (0/0.1/0.2/0.23/0.26/0.3/0.4/0.5% W/W).

0.5 mL diluted serum was mixed with 0.5 mL 2x sample buffer. The mixture was then heated at 45 ºC for 15 min before 15 μm was loaded into gel well.

SDS-PAGE running parameters used were: 200 V/20 mA with controlled voltage which changed to 200 V/17 mA after 45 min when running finished.

6.1.8. Visual observation of emulsions

0.25 mL of 1% Oil Red O was added into 100 mL emulsion, 30 mL of dyed emulsions were transferred into flat-bottomed tubes. Emulsion separation was observed and recorded at time points of: 0, 1, 2, 24, 48 hours after multilayer emulsions were made.

6.1.9. Confocal microscopy scanning

After 20 min of incubation, dyed emulsions containing 2 (% V/V) nile red and 6 (% V/V) fast green was examined using a Leica SP5 DM6000B Scanning Confocal Microscope (Leica Microsystems, Germany), with a ×40 objective. Excitation and measurement were conducted at 488 nm and 570-676 nm for nile red; 633 nm and 498-555 nm for fast green respectively.

6.2. Results

6.2.1. Emulsion droplet size

At neutral pH, the droplet size of caseinate-stabilized emulsions increased significantly once the lysozyme concentration exceeded 0.1 wt.% , whereas droplet distributions were independent of lysozyme addition at pH of 3.

Lysozyme addition increased emulsion droplet size at neutral pH (Fig. 9.). The initial addition of 0.1 wt.% of lysozyme did not cause a significant change in D[3,2], but D[4,3] increased slightly (0.50-4.04 μm), indicating larger aggregation appeared as soon as lysozyme was added to the system. Further increases of lysozyme concentration led to a strong shift to larger particle sizes in the emulsions, until the size peaked when lysozyme concentration reached 0.3 wt.% . Increases of lysozyme above 0.3 wt.% to 0.4 wt.% resulted in a slight decrease in the values of both D[4,3] and D[3,2], with further increases up to 0.5 wt.% having no significant effect. It also can be seen that the rates of particle size increase differed according to lysozyme concentration. Droplet

sizes increased most dramatically when lysozyme concentration shifted from 0.2 wt.% to 0.26 wt.% .

Fig. 10. shows there were little effects of lysozyme concentration on droplet size distribution of Na-CN stabilized emulsions under acid pH. Increasing lysozyme concentration did not contribute to significant changes in D[3,2] value. Similarly, D[4,3] values remained consistent at lysozyme concentrations above 0.1 wt.% . This initial 0.1 wt.% addition of lysozyme resulted in a significant decrease in D[4,3] value.

There were also differences between droplet sizes of primary emulsions at acid and neutral pH. In the absence of lysozyme, the emulsions under the different pH conditions shared the same D[3,2] values, whereas the D[4,3] values of emulsions at acid pH were higher than those of emulsions at neutral pH, where values of D[4,3] and D[3,2] were the same, meaning more aggregation was formed in the primary emulsions at acid pH.

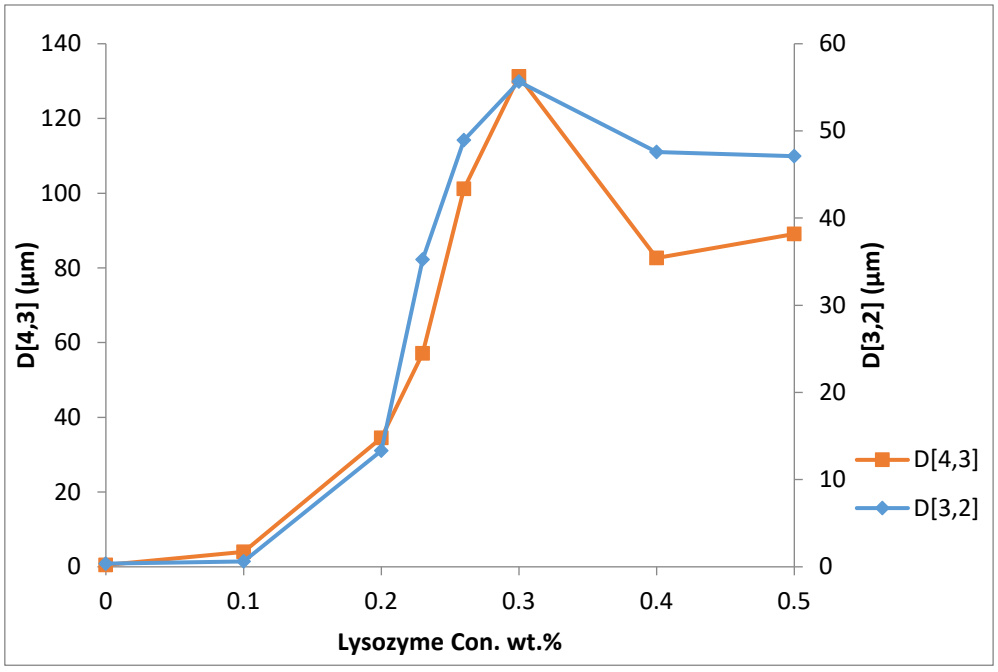

Figure 9. Zeta-potential of multilayer emulsions (10 wt.% oil, 0.4 wt.% caseinate, pH 6.8±0.2, 22 °C) containing lysozyme 3 replicates

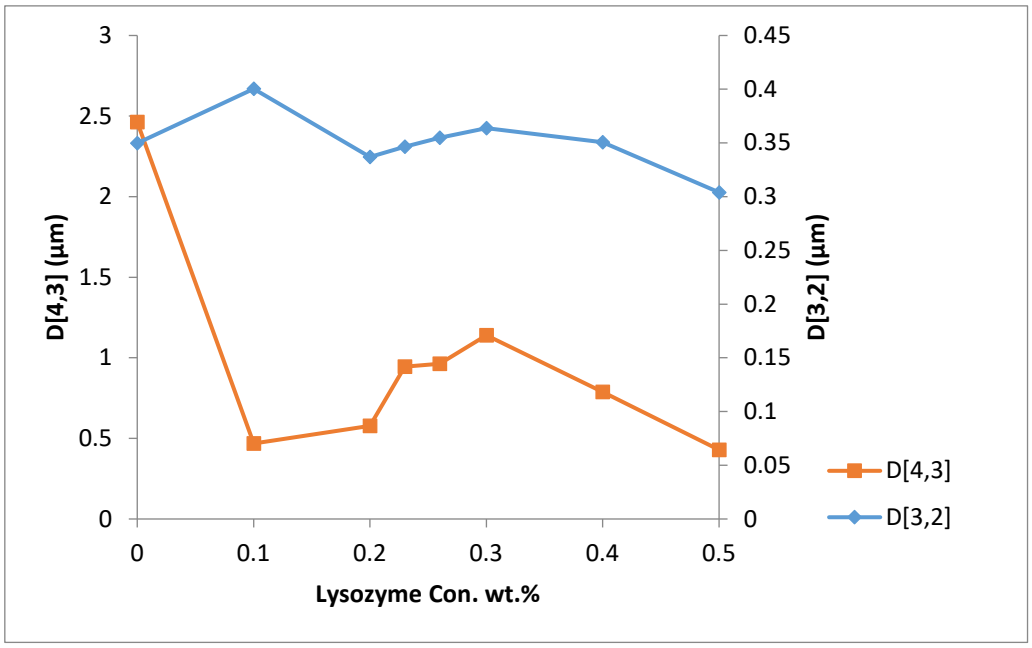

Figure 10. Particle size of multilayer emulsions (10 wt.% oil, 0.4 wt.% caseinate, pH 3.3±0.3, 22 °C) containing lysozyme 3 replicates

6.2.2. Zeta-potential

At neutral pH, the surface charge characteristics of caseinate-stabilized emulsions increased as more lysozyme was present in the system. This is in stark contrast to that of acidified emulsions which stayed relatively stable regardless of lysozyme addition.

Fig. 11 shows the emulsion zeta-potential change plotted against lysozyme concentration. The zeta-potential constantly shifted towards more positive as lysozyme concentration increased. However, the increase consisted of three stages according to change rates: firstly, during the initial addition of 0.2 wt.% of lysozyme, the increase rate is the lowest; at lysozyme concentrations between 0.2 wt.% and 0.4 wt.% , the zeta-potential rose most dramatically; after which in the third stage the positive increases were smaller in size. Fig. 12 shows the interface charge of emulsions were insensitive to lysozyme concentrations at acid pH.

At neutral pH, the standard errors were higher after lysozyme concentration exceeded 0.3 wt.% , indicating a more diverse interface after that lysozyme concentration. At acid pH, however, the standard errors were almost unchanged for all experiments, suggesting again, lysozyme addition did not affect caseinate-stabilized emulsions at acid pH.

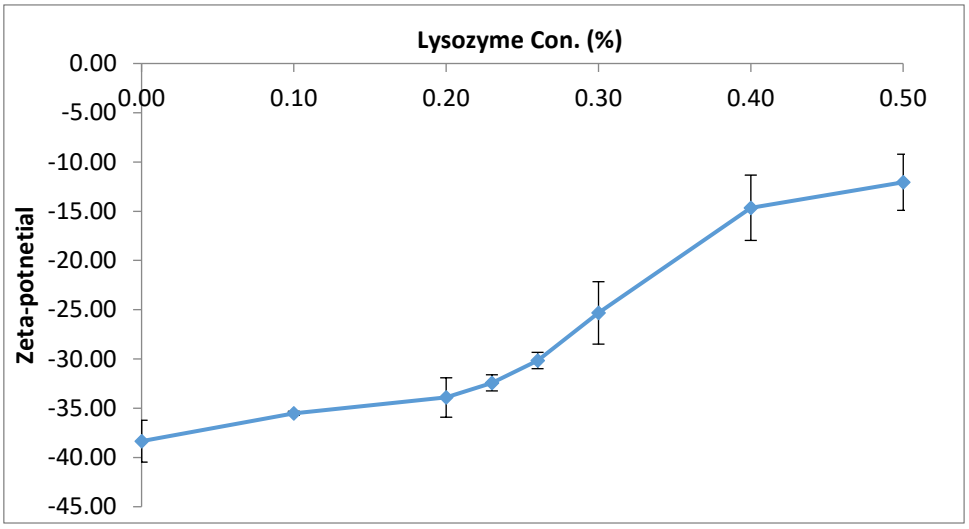

Figure 11. Zeta-potential of multilayer emulsions (10 wt.% oil, 0.4 wt.% caseinate, pH 3.3±0.3, 22 °C) containing lysozyme 3 replicates

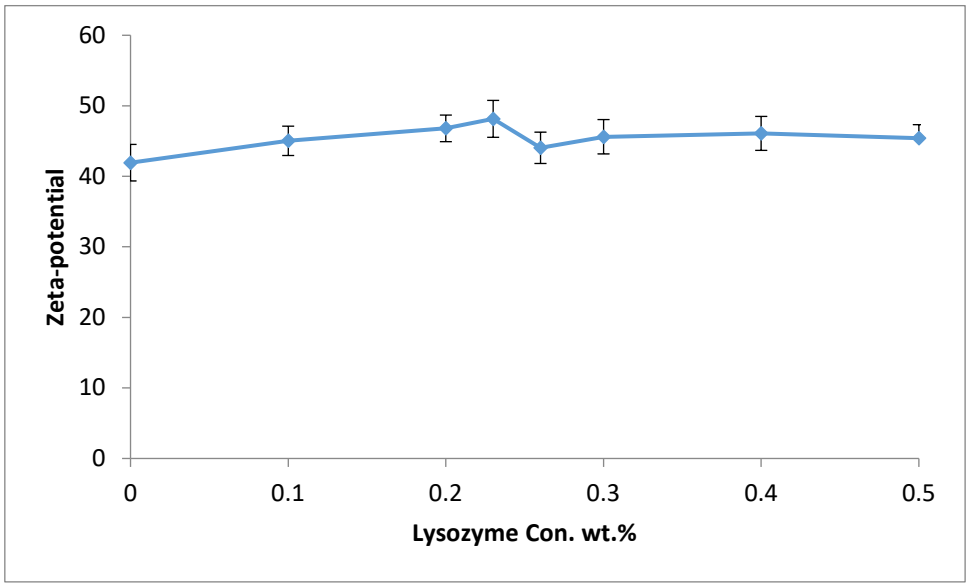

Figure 12. Zeta-potential of multilayer emulsions (10 wt.% oil, 0.4 wt.% caseinate, pH 3.3±0.3, 22 °C) containing lysozyme 3 replicates

6.2.3. Protein concentration of phases

Under the both pH conditions, addition of lysozyme resulted in a rise of protein concentration in only one phase (cream phase for neutral pH, serum phase for acid pH), while the protein content of the other phase did not change significantly. The change of protein concentration as a function of lysozyme addition at neutral pH is shown in Fig. 13. The serum protein remained unchanged at around 0.015 wt.% despite of the increase of lysozyme concentration, whereas concentration at interface increased proportionally with increases in lysozyme. This indicates that all of the added lysozyme was associated with the droplet interface. Fig. 14 shows the change of protein content in the phases of acidified emulsions. Under these conditions, the interfacial protein content was independent to lysozyme concentration changes, but the serum protein content increased gradually as more lysozyme was added.

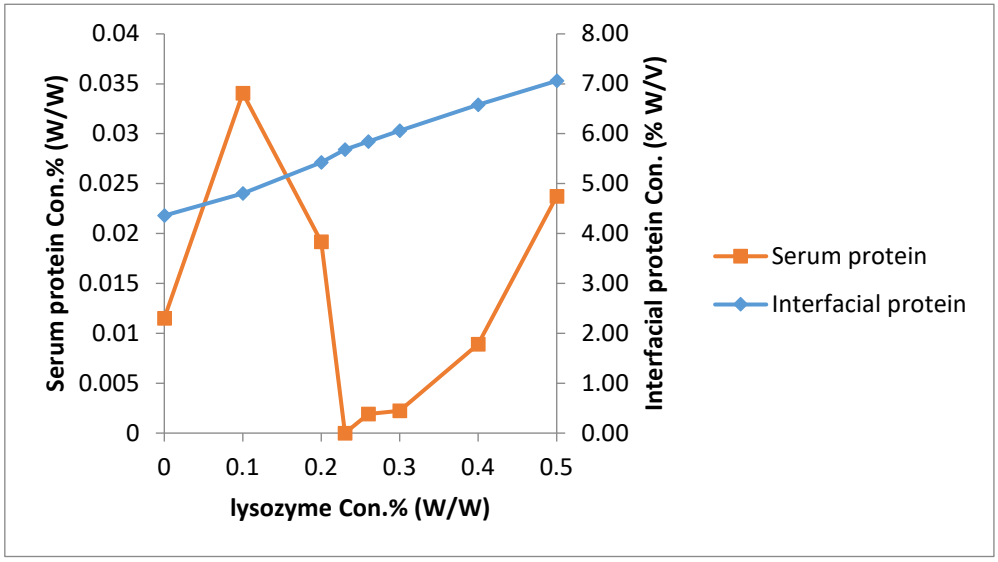

Figure 13. Protein content of multilayer emulsions (10 wt.% oil, 0.4 wt.% caseinate, pH 6.8±0.2, 22 °C) containing lysozyme 2 replicates

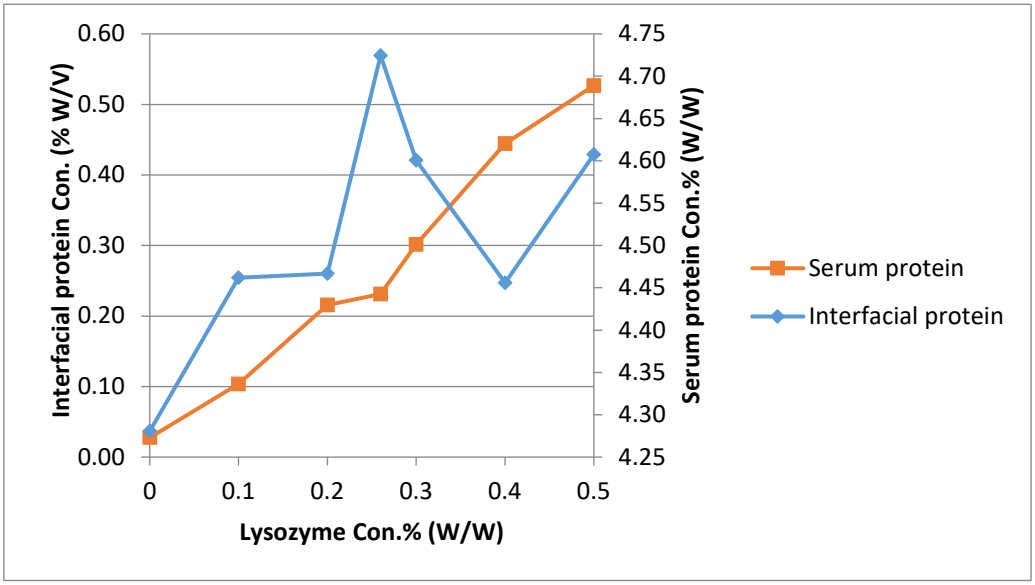

Figure 14. Protein content of multilayer emulsions (10 wt.% oil, 0.4 wt.% caseinate, pH 3.3±0.3, 22 °C) containing lysozyme 2 replicates

6.2.4. SDS-PAGE analysis of serums of acidified mixed emulsion

The SDS-PAGE (Fig. 15) showed that there was only one species of protein, lysozyme, present in the acidified emulsions serum.

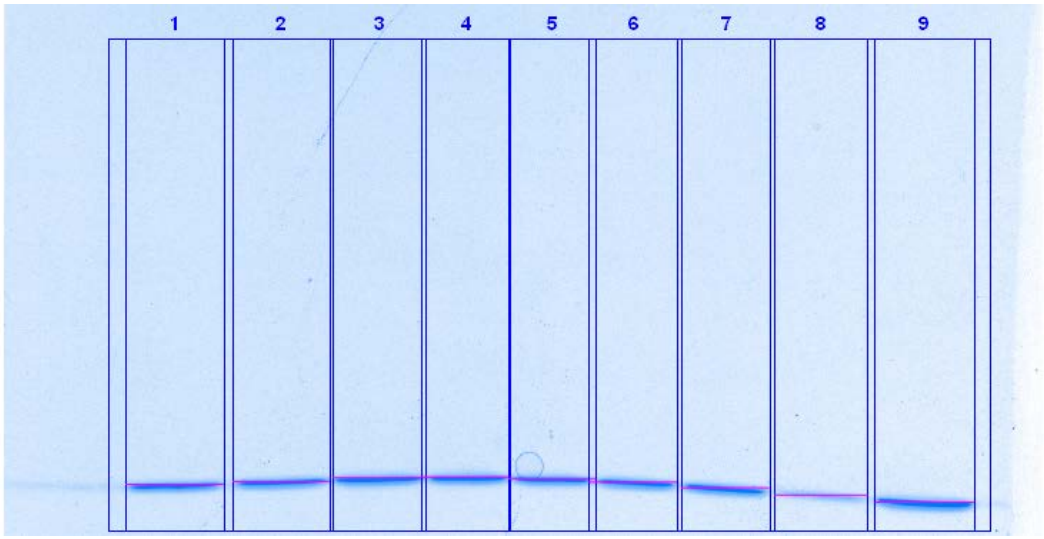

Figure 15. SDS-PAGE results. Well 1-8: serum of acidified emulsions containing 0.5, 0.4, 0.3, 0.26, 0.23, 0.2, 0.1, 0 wt.% lysozyme. Well 9: 0.1% lysozyme solution

6.2.5. Visual separation

The creaming stability of acidified emulsions was not affected by the presence of lysozyme. In contrast lysozyme addition to emulsions at neutral pH resulted in destabilization.

Fig. 16 and Fig. 17 show phase separation of emulsions with different lysozyme concentrations at neutral and acid pH. Oil phase were shown red, while serum was translucent. It can be concluded that presence of lysozyme resulted in phase separation of emulsions at neutral pH, and increasing lysozyme concentration depleted more oil droplets from the serum phase. Nevertheless, no phase separation was observed to occur in acidified emulsions at any lysozyme concentration.

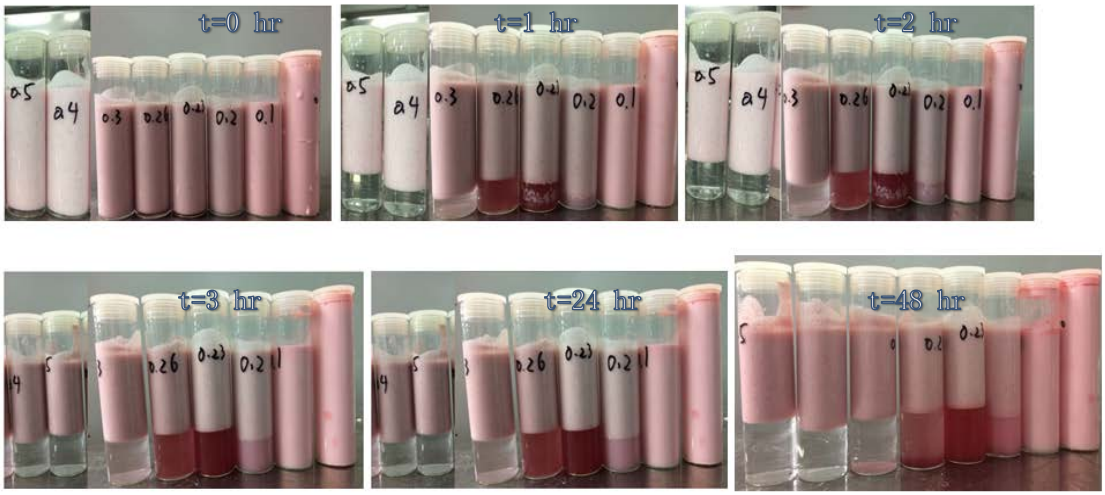

Figure 16. Visual phase separation of multilayer emulsions (10 wt.% oil, 0.4 wt.% caseinate, pH 6.8±0.2, 22 °C) containing different concentrations of lysozyme (from right to left 0, 0.1, 0.2, 0.23, 0.26, 0.3, 0.4, 0.5% W/W) at different time point (hours)

Figure 17. Visual phase separation of multilayer emulsions (10 wt.% oil, 0.4 wt.% caseinate, pH 3.3±0.3, 22 °C) containing different concentrations of lysozyme (from left to right 0, 0.1, 0.2, 0.23, 0.26, 0.3, 0.4, 0.5% W/W) at different time point (hours)

6.2.6. Confocal microscopy scanning

Under confocal microscopy (refer Fig.18 to 22) the oil droplets are shown as white dots, and proteins are shown as the grey areas. Under the neutral pH conditions, as more lysozyme was present in the system, aggregation was observed, and the extent of aggregation increased with increases in lysozyme addition. Although aggregation increased there was no significant increase in the amount of visible larger droplets as lysozyme concentration increased, indicating droplet flocculation is the mechanism for destabilisation rather than coalescence. The evidence suggesting aggregation rather than coalescence is based on the observations regarding the edge of the aggregates. On inspection, it is evident that the edges are fractal in nature rather than smooth which is what one would expect from coalescence alone. Under acid pH conditions, the droplet aggregation states of emulsions containing 0 wt.% and 0.5 wt.% lysozyme did not differ significantly. Additionally, more aggregated droplets were spotted in the primary emulsions at acid pH (Fig. 21) than that at neutral pH (Fig. 18).

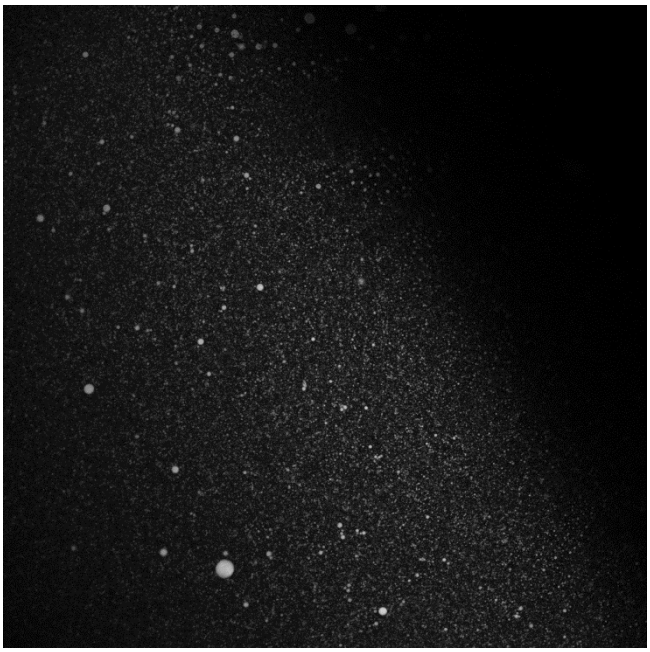

Figure 18. Confocal microscopy scanning of emulsions (10 wt.% oil, 0.4 wt.% caseinate, 0 wt.% lysozyme, pH 6.8±0.2, 22 °C)

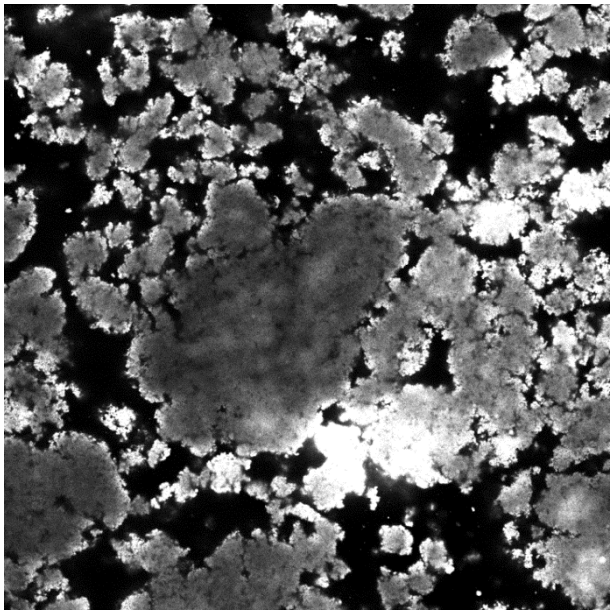

Figure 19. Confocal microscopy scanning of emulsions (10 wt.% oil, 0.4 wt.% caseinate 0.3 wt.% lysozyme, pH 6.8±0.2, 22 ºC)

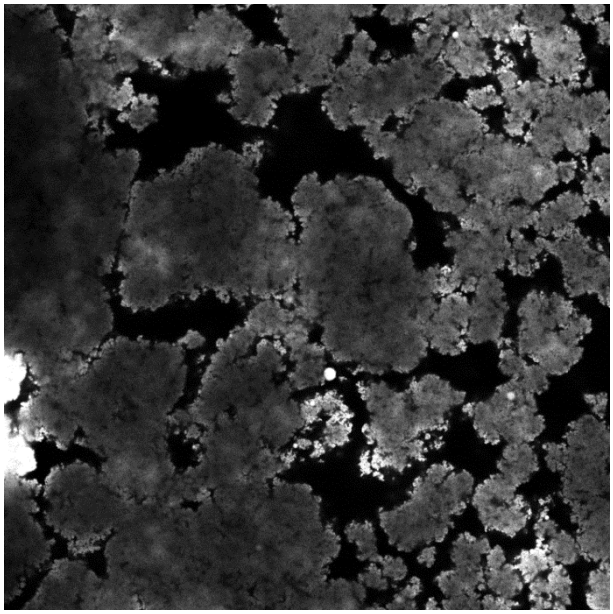

Figure 20. Confocal microscopy scanning of emulsions (10 wt.% oil, 0.4 wt.% caseinate 0.5 wt.% lysozyme, pH 6.8±0.2, 22 ºC)

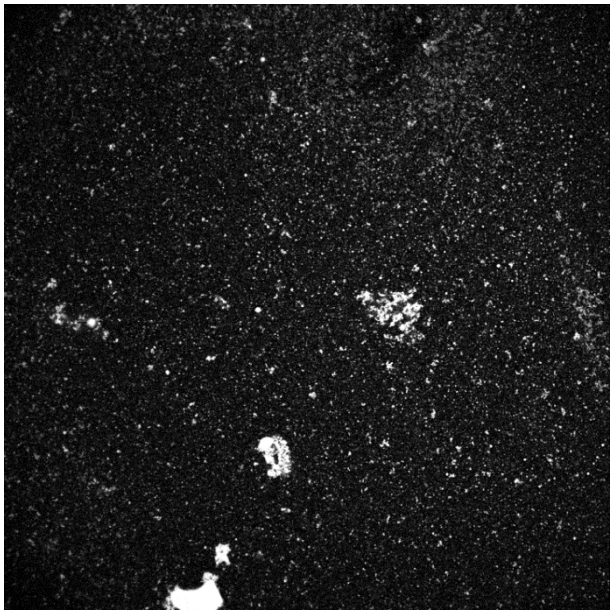

Figure 21. Confocal microscopy scanning of emulsions (10 wt.% oil, 0.4 wt.% caseinate 0 wt.% lysozyme, pH 3.3±0.3, 22 ºC)

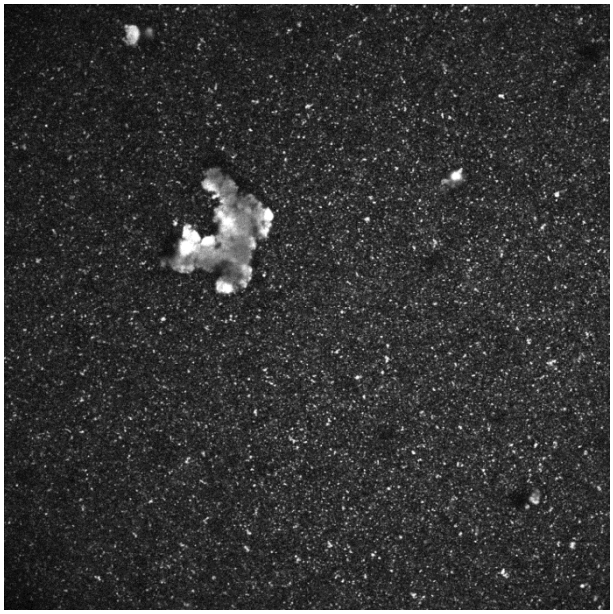

Figure 22. Confocal microscopy scanning of emulsions (10 wt.% oil, 0.4 wt.% caseinate 0.5 wt.% lysozyme, pH 3.3±0.3, 22 ºC)

6.3. Discussion

Lysozyme associates with caseinate-stabilized emulsions through electrostatic interaction with interfacial caseinate, which happens between solution lysozyme and caseinate [110, 114]. As the most significant change during acidification of the interface is the charge of adsorbed caseins, which switches from negative to positive, this alters the forces between caseins and lysozyme from attractive to repulsive. Therefore, in the present study while almost all lysozyme was associated with the interface at neutral pH there was no significant interaction at acid pH.

At neutral pH, the presence of lysozyme in sodium caseinate stabilized emulsions causes aggregation of the oil droplets. The likely mechanism for lysozyme destabilising neutral pH caseinate emulsions is screening casein charges. At low concentrations (0.1 wt.% lysozyme) the degree of aggregation was small. While lysozyme will still interact with caseinate, due to its insufficient amount, the degree of charge screening on the caseinate molecules is probably inadequate to cause significant bridging between caseinate stabilised oil droplets. The remaining exposed charges could provide enough repulsion to maintain a relatively stable emulsion. Therefore, no flocculation occurred.

When lysozyme concentration exceeded 0.2 wt.% , the amount of lysozyme is sufficient to cause destabilization, but not enough to aggregate all the caseins in the system, therefore stable oil droplets can still be observed in the serum layer. The complete depletion of oil droplets in serum layer happens at lysozyme concentration of 0.4 wt.% , indicating the presence of an excessive amount of positively charges of lysozyme, thus further addition of lysozyme had no effects on emulsion appearance.

The destabilization when lysozyme concentration increased from 0.1 to 0.2 wt.% was accompanied with a drop in the zeta-potential (-35.5 to -34), and one hypothesis that could explain this is that the aggregation is due to the decrease in zeta potential and that this causes insufficient electrostatic repulsion for the droplets to remain stable. An alternative hypothesis is that bridging flocculation is occurring and that the bridging is

mediated by the lysozyme. The first hypothesis can be disputed, as it is known that sodium caseinate stabilized emulsions stay stable against sodium chloride [126] . The addition of sodium chloride only screens the surface charge of caseinate stabilized emulsions. Additionally, β-casein stabilized emulsions are known to remain stable when the zeta-potential is as low as +5 because of steric repulsion, and emulsions produced with sodium caseinate, due to its high content of β-casein, can also maintain stability with a low interface charge [127, 128]. Therefore, at low lysozyme concentration, destabilization of caseinate-stabilized emulsions is likely to be caused primarily by bridging flocculation.

This bridging flocculation effect is decreased as more lysozyme is present in the system. As discussed above based on the zeta size data, 0.4 wt.% lysozyme is close to the critical point where the positively charged residues of lysozyme are just sufficient for maximum shielding of the negatively charged residues, and further increases of lysozyme provide more available positive residues in system, which will decrease the amount of caseins one lysozyme molecule associates with. In this case, bridging flocculation effects decrease and thus the degree of aggregation of droplets decreases as well. Therefore, there was a drop of both values of D[4,3] and D[3,2] when lysozyme concentration shifted from 0.3 wt.% to 0.4 wt.% . This behaviour is consistent with a model built by a previous study in the literature, where bridging flocculation causes destabilization at low secondary polyelectrolyte concentration, and the effects are diminished at high concentration. [1].

At neutral pH, the increase of zeta-potential on the addition of lysozyme is attributed to the screening of charges on casein while the increase of exposed positively charged residues does not contribute to interface charge significantly. The highest increase rate of zeta-potential happens when bridging flocculation of lysozyme dominated, where one lysozyme molecule interacts with highest number of casein, in the present study at 0.2-0.4 wt.% . Therefore, in the present study, the newly introduced positively charged residues, due to further increases in lysozyme concentration (>0.4% w/w), increased

the amount of positively charged residues at the interface, of which a larger ratio was exposed compared to that at lysozyme concentrations of 0.2-0.4 wt.% , did not incur a similar or higher increase of the zeta-potential.

As multilayer emulsions containing polysaccharides usually go through stable-unstable-stable as the concentration of polysaccharides increases[1], it may be possible that such a phenomenon might occur with the addition of lysozyme. However, the range of lysozyme addition >0.4% w/w is not enough to evaluate whether stable emulsions could be created again. The destabilization mechanism of polysaccharides at low concentrations is considered similar to lysozyme in the present study. The re-stabilization is due to electrostatic or steric repulsion due to the increasing presence of polysaccharides at interface, plus the increase of viscosity by resulting from the higher concentration of polysaccharides in the serum phase, which hinders the movement of droplets[51].

At acid pH, caseinate stabilized emulsions stayed almost unaltered against addition of lysozyme. Under acidic conditions, lysozyme and adsorbed casein possess same charge, making electrostatic interaction between them repulsive, and the poor emulsifying ability of lysozyme prevents the adsorption of lysozyme onto O/W interface directly. Consequently, lysozyme does not associate with, or change the interface of caseinate stabilized emulsions at acid pH. This hypothesis is supported by the results of visual observation, zeta-potential measurement, microscopy scanning and protein content analysis of phases.

Addition of lysozyme can disrupt the flocculation of emulsions containing caseinate at acid pH. In the present study, the D[4,3] particle size dropped in the presence of lysozyme. Due to its small size and high charge, lysozyme might be able to move into interspatial areas between flocculated fat droplets, and provide extra electrostatic repulsion between flocculated droplets.

7. Conclusions

At low lysozyme to casein ratios, complexes formed by lysozyme and caseinate at neutral pH are not suitable to create a stable emulsion system. At low lysozyme to caseinate ratios, the majority of complexes stay stable in solution, but generates unstable emulsions. On the other hand, when complexes consist of large amounts of lysozyme, the aggregation is precipitated.

Emulsions with a caseinate primary interface containing lysozyme cannot be a stable multilayer system. At neutral pH, the addition of small amounts of lysozyme leads to destabilization of caseinate-stabilized emulsions. Increasing the concentration of lysozyme up to 0.5% w/w did not promote stabilization. At acid pH, on the other hand, lysozyme does not change properties of caseinate interface layer.

Areas of interest for further study include:

1) Investigate complexes with high lysozyme to casein ratio as emulsifiers (>0.5% w/w) and the properties of emulsions formed with them;
2) Investigate the effects of caseinate on primary emulsions stabilized by lysozyme.

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