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Emulsifying Properties of Interfacial Components of Coconut Oil Bodies

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Abstract

Oil bodies are organelles in plant that store triacylglycerol (TAG) in plants. Some oil bodies exhibit remarkable physical and chemical stability to against coalescence and lipid oxidation due to their unique interfacial layer. These interfacial biomaterials present themselves as ideal materials for encapsulation and have potential applications in food, pharmaceutical and cosmetic formulations. This study compared the emulsifying properties of biomaterials obtained from coconut oil body membrane (COBM) and coconut skimmed milk extracts (CSME) and investigated the structure of resulting COBM and CSME emulsions.

Coconut oil body membrane (COBM) and coconut skimmed milk extracts (CSME) were extracted from freshly prepared coconut milk (around 20%, w/w, fat). The properties of CSME and COBM such as solubility, isoelectric pH and their ability to reduce the interfacial tension between water and soybean oil was characterized by tensionmeter. The CSME and COBM (0.2, 0.4, 0.6 and 0.8%, w/w, final protein concentration) were used for preparing soybean oil-in-water emulsions (20%, w/w) by microfluidizer. The physicochemical characteristics of the CSME and COBM emulsions at different pH (2-8) and NaCl (0-500 mM) conditions were investigated by using dynamic light scattering techniques and confocal laser scanning microscopy. Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) was used to characterize the proteins composition of the 2 extracts (CSME and COBM) and the proteins composition of droplet interfacial proteins of CSME and COBM emulsions. To investigate the surface composition of CSME and COBM emulsions, the surface of the droplet of CSME and COBM emulsions was perturbed by the enzymes pepsin, trypsin and phospholipase A₂ (PLA₂), separately.

The SDS-PAGE analysis of two extracts showed distinct differences in the protein composition of CSME and COBM. The isoelectric points of CSME and COBM solutions were between pH 4 and 5. Both extracts lowered the interfacial tension between water and oil but the extent of decrease in surface tension was greater for COBM than that for CSME, indicating that COBM was more surface active than CSME.

The particle size of CSME emulsion decreased with an increase in protein concentration, while the effect of protein concentration on particle size was less pronounced in COBM emulsions. Compared with CSME emulsion, COBM emulsion had a smaller particle size with less degree of flocculation and was more stable during storage. These results suggest that COBM had the better emulsifying capacity than CSME. The CLSM images revealed that the droplet surface of CSME and COBM emulsions consisted of both protein and phospholipids. Both pepsin and phospholipase A₂ treatment of CSME and COBM emulsions lead to the coalescence, which indicates the possible droplet interfacial layer structures of CSME and COBM emulsions are similar, that both phospholipids and protein sequences with aromatic and hydrophobic residues present at the interface. The SDS-PAGE analysis of the droplet surface proteins of CSME and COBM emulsions revealed that not all proteins in CSME and COBM were adsorbed on the droplet surface of CSME and COBM emulsions. The surface protein composition of COBM emulsion was similar to that of natural coconut oil body.

The effect of pH on CSME and COBM emulsions revealed that the isoelectric points of both emulsions were close to each other (around pH 4.7 and 4.5 for COBM and CSME respectively). In addition, both COBM and CSME emulsions were stable at high pH (pH > pI) but had different behaviour below pI. While the COBM emulsion flocculated at pH near pI and the CSME emulsion showed coalescence at pH ≤ pI.

In the presence of salt, CSME and COBM emulsions were still negatively charged, even at 500 mM NaCl, indicating that 500 mM NaCl was unable to screen all the charges on the droplet surface. Flocculation occurred in COBM emulsion with the increase in NaCl concentration. No coalescence was observed in CSME emulsion at all given salt concentrations. This work shows that COBM emulsion has better stability against changes in pH than CSME emulsion.

Both COBM and CSME were successfully stabilized emulsions, which indicates that COBM and CSME may be suitable for use as a food emulsifier. COBM seems to be a better emulsifier material, since it can form emulsions with smaller droplet size and the flocculation occurred at low pH and high salt conditions may not be a major problem in some food, such as sauces and yoghurts.

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List of Abbreviations

| | |
|------------------|---|
| CCP1 | Coconut cream protein (isoelectric precipitation) |
| CCP2 | Coconut cream protein (freeze-thaw) |
| CLSM | Confocal laser scanning microscopy |
| COBM | Coconut oil body membrane |
| CSME | Coconut skimmed milk extracts |
| CSPC | Coconut skimmed milk protein concentrate |
| CSPI | Coconut skimmed milk protein isolate |
| $d_{4,3}$ | Average volume-weighted diameter |
| $d_{3,2}$ | Average surface-weighted diameter |
| FG | Fast Green |
| HLB | Hydrophilic-lipophilic balance |
| MFGM | Milk fat globular membrane |
| NR | Nile Red |
| OB | Optical density |
| pI | Isoelectric point |
| PLA ₂ | Phospholipase A2 |
| POB | Protein of oil body |
| Rd-DHPE | Lissamine TM rhodamine B |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide electrophoresis |
| SGF | Simulated gastric fluid |
| SIF | Simulated intestinal fluid |
| TAG | Triacylglycerol |
| w/w | Weight/weight |
| w/v | Weight/volume |
| v/v | Volume/volume |

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1. Introduction

1.1 Introduction

Recently, the oil bodies from plants have received appreciable attention, since they are relatively stable under various conditions. The high structural stability of the oil bodies could be attributed to their interfacial membrane (Acevedo et al., 2014; Nikiforidis, Matsakidou, & Kiosseoglou, 2014). These interfacial biomaterials present themselves as ideal materials for encapsulation and have potential applications in food, pharmaceutical and cosmetic formulations. In the food industry, some oil body interfacial materials (such as milk fat globule membrane and lecithin) have already been successfully used as food emulsifiers. With increasing demand for foods with health benefits and functional proteins from plant sources, the oil body membrane materials present themselves as high-value functional ingredients.

Coconut milk or coconut cream is an aqueous extract of coconut endosperm and widely consumed in many tropical areas. The coconut oil in the coconut fruit exists as spherical droplets or oil bodies that are stabilized by phospholipids and proteins. The manufacture of virgin coconut oil generates a byproduct stream, which is rich in coconut oil body membrane materials (COBM). This COBM can be extracted using a ‘clean and green’ process without the use of toxic solvents resulting in a natural emulsifier suitable for use in food formulations. Furthermore, understanding the properties of these biomaterials may help in exploring their applications and contribute to the economies of New Zealand’s neighbouring countries in the Pacific, that grow coconuts.

The main objective of this study is to explore new emulsifier materials extracted from coconut oil body interfacial membrane. This study investigated the properties of emulsions formed from coconut skimmed milk extract (CSME) and coconut oil body membrane material (COBM) and compared the structure and physicochemical stability of the by CSME and COBM emulsions.

The Chapter 2 provide a brief overview of the principle of emulsification and the factors affect the stability of emulsions. The structure of oil bodies from plants and properties of natural coconut oil bodies are also discussed in Chapter 2. The protocols used to characterize the emulsions are described in Chapter 3. The composition and the properties

of the CSME and COBM are discussed in Chapter 4. In Chapter 5, the effects of different concentrations of these extracts on the characteristics of emulsions are studied. In order to get detailed information about the characteristics of the interfacial membrane, the emulsion droplets were perturbed by treatment with enzymes pepsin, trypsin and phospholipase A₂. The information derived from the enzymatic studies was used to get details about the distribution of interfacial components on droplet surfaces. Chapter 6 investigates the effects of pH (2-8) and ionic strength (0 to 500 mM NaCl) on the stability of the CSME and COBM emulsions at two protein concentrations.

1.2 Outline of thesis

The schematic showing the layout of this thesis is shown in **Figure 1.1**:

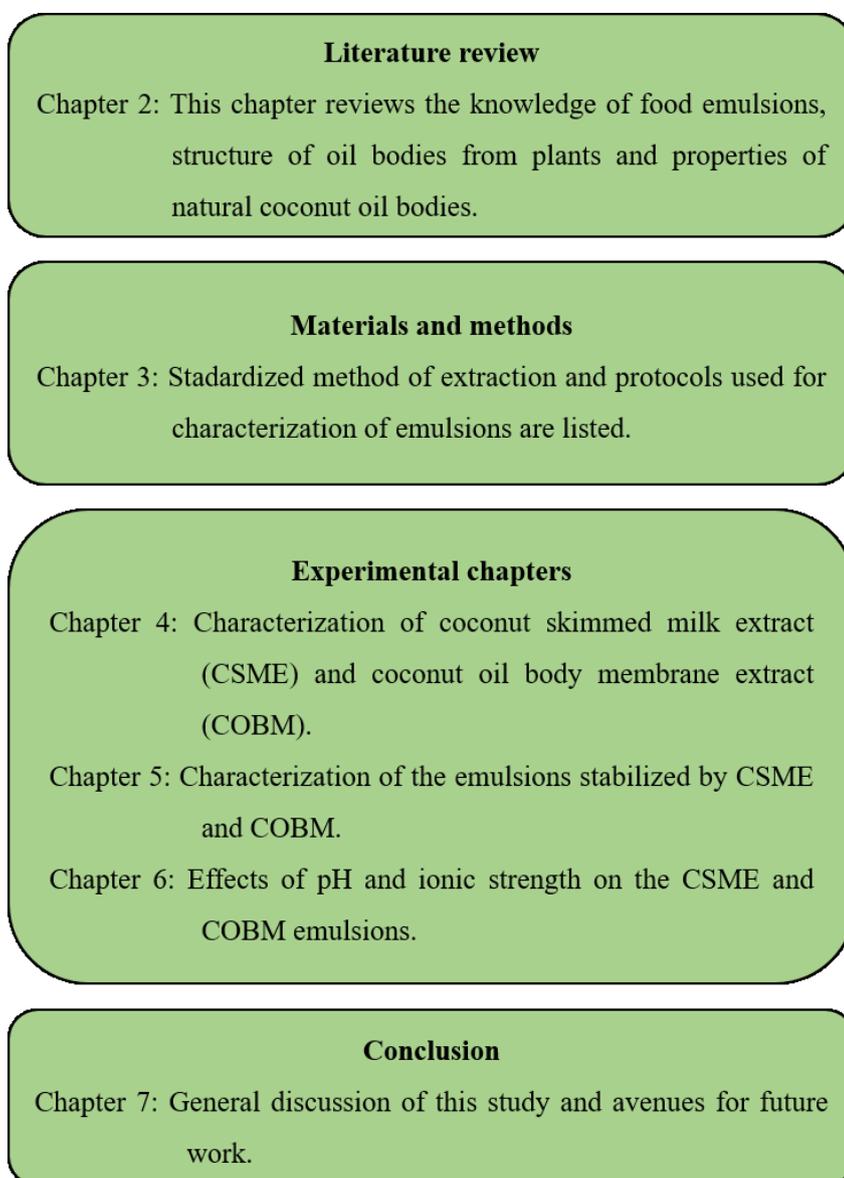


Figure 1.1 Overview of chapters in this thesis.

2. Literature review

2.1 Food emulsions

An emulsion is defined as a dispersion system in which at least one liquid phase (dispersed phase) is dispersed in another immiscible liquid phase (continuous phase) (McClements, 2015; Santana, Perrechil, & Cunha, 2013). The dispersed phase is present as small spherical droplets, which have a diameter ranging from 0.1-100 μm in the continuous phase. Since the increase of oil and water interfacial area affects a number of ingredient functionalities such as solubility, encapsulation and bioavailability, fine droplet size emulsion (nano-emulsion) has received significant interest recently (Cortés-Muñoz, Chevalier-Lucia, & Dumay, 2009; McClements, 2015; Santana et al., 2013; Walstra, 2002).

Generally, in a food emulsion, two immiscible phases are usually water and oil. Emulsions can be classified into three groups based on the nature of the dispersed and continuous phases. Oil in water emulsion (O/W emulsions) (Figure 2.1, A) are emulsions in which the oil is dispersed in and water which forms a continuous phase. Water in oil emulsions (W/O emulsions) (Figure 2.1, B) refers to emulsions in which water is the dispersed phase and oil acts as a continuous phase. Finally the above two types of emulsions (W/O or O/W) can act as dispersed phases themselves in an oil or aqueous medium resulting in multiple emulsions oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) (Figure 2.1, C and D) (Kim, Decker, & McClements, 2006; McClements, 2015; Santana et al., 2013). Some examples of emulsions in the food system are listed in Table 2.1. In this review, literature pertaining to oil-in-water emulsions is discussed.

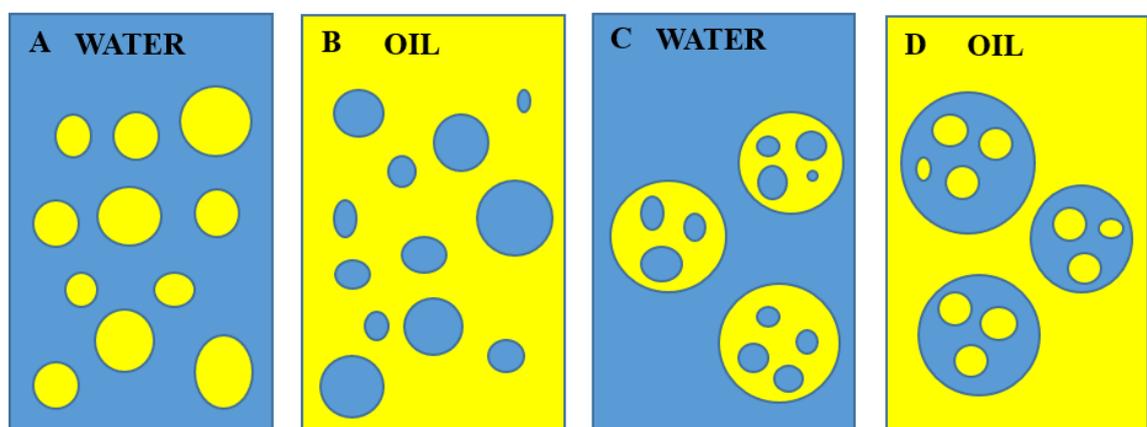


Figure 2.1 Illustration of different types of emulsions

Table 2.1 Examples of emulsions in food systems

| Food | Type | Stabilization Mechanism |
|----------------------|-------------|---|
| Milk | O/W | Protein and phospholipids membrane |
| Cream | O/W | Protein-membrane and stabilization of air |
| Ice cream | A+O/W | Protein-membrane and ice network |
| Butter and margarine | W/O | Fat crystal network |
| Mayonnaise | O/W | Polysaccharide |

2.1.1 Emulsification

Emulsification is defined as the process of mixing two immiscible phases into a uniform emulsion. In general, emulsification methods are divided into two groups based on the energy input required for emulsification. These are high-energy methods and low-energy methods (spontaneous formation) (McClements, 2015). High-energy methods are usually used to produce most of food emulsions products, due to its ability to create strong disruptive forces to disrupt dispersed phase into continuous phase (McClements, 2015; Santana et al., 2013; Silva, Cerqueira, & Vicente, 2012).

In most instances, emulsion formation starts at the preparation of two immiscible phase, in which water-soluble and oil-soluble ingredients (including emulsifiers) are dissolved in water and oil phase, respectively. When two immiscible phases meet together, they tend to minimize the contact area and separate into a top oil layer and a bottom water layer, which is the most thermodynamically stable state. Emulsification requires a large amount of free energy, provided by homogenization equipment, and sufficient emulsifier. Free energy would increase the water-oil interface area and disrupt the oil and water phases (Singh & Ye, 2013). The emulsifiers could rapidly adsorb on the surface of the disrupted droplets to decrease the interfacial tension and stabilize the emulsion. The details of emulsifiers are discussed later (Dickinson, 1992; McClements, 2015).

2.1.2 Homogenization equipment

Homogenization could be defined as a process of intermingling two immiscible phases into an emulsion. The equipment used to execute the homogenization is called

homogenizers (McClements, 2015). The mechanism of homogenization is that the coarsely mixed water and oil solution (primary emulsion), which contain surface active agents, is forced to pass through a narrow slit at a high-pressure condition which can lead to an intense shear flow. During this process, homogenizers introduce a huge amount of mechanical energy to disrupt the dispersed phase into small droplets. Hence, the interfacial area of oil and water is increased (McClements, 2015). At the same time, emulsifiers, which have amphiphilic structures, adsorb at newly generated droplet interface to create and stabilize a fine droplets dispersed emulsion system (Dickinson, 2003; McClements, 2015).

The purpose of primary homogenization is to generate a coarse emulsion, which has large droplets, while the secondary homogenization is to reduce the droplet size. Depending on different purpose and the droplet size desired, different equipment may be selected to generate mechanical force, these include colloid mills, high-pressure valve homogenizers, high speed mixers, ultrasonic homogenizers and microfluidization homogenizers (Santana et al., 2013; Schultz, Wagner, Urban, & Ulrich, 2004; Walstra, 2002). In this review, only high-pressure valve homogenizer and microfluidizer are discussed.

High-pressure valve homogenizer

High shear mixer is usually used for primary homogenization, followed by inputting into high-pressure valve homogenizer (Figure 2.2) (McClements, 2015). For dairy systems, the primary emulsions are introduced into two-stage high-pressure valve homogenizers, which are assembled with two consecutive valves. High pressure is set up at first valve to form fine droplets and relatively low pressure is introduced at the second valve to disrupt flocculation created in the first stage.

The advantage of high-pressure valve homogenizer is that it is suitable to produce fine emulsion droplets with narrow emulsion droplets size distribution (minimum droplet size at 0.1 μm) (Santana et al., 2013; Schultz et al., 2004; Stang, Schuchmann, & Schubert, 2001). Compared with colloid mill, high-pressure valve homogenizer can achieve the better effect on reducing droplet size.

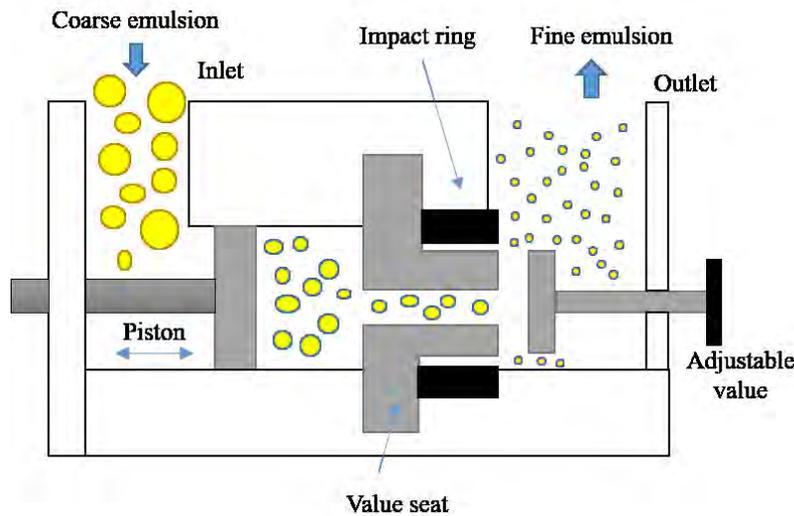


Figure 2.2 Schematic showing the flow of the product through the homogenizer

Microfluidizer

In a microfluidizer, the emulsion fluids are accelerated to high speed by using a pressure pump and introduced into streams. When the streams collide with each other, the collisions between droplets result in intense disruptive forces that cause droplet disruption and dispersion (Lee & Norton, 2013; McClements, 2015; Panagiotou, Mesite, & Fisher, 2009). Microfluidizer could be design as (A) single or (B) double inlets for pre-mixed emulsions and two immiscible phases (Figure 2.3).

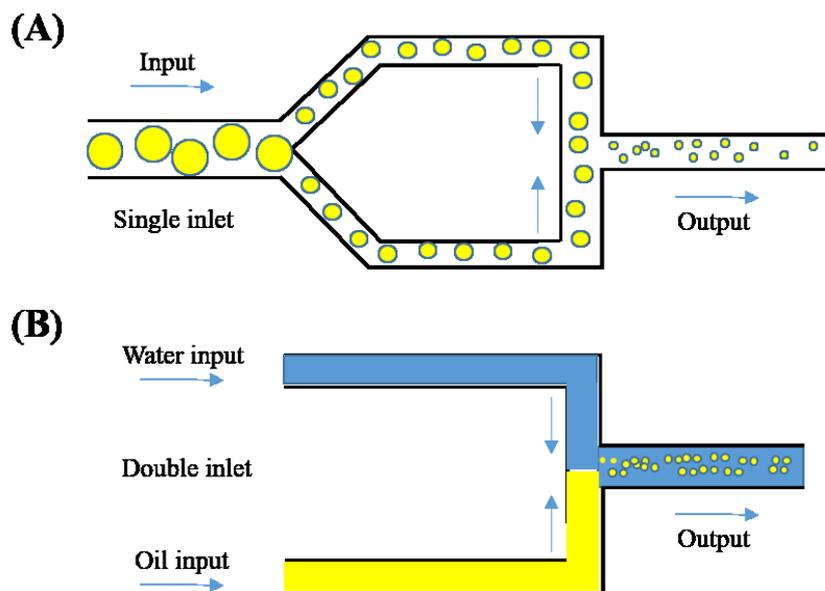


Figure 2.3 Single and double inlet model of microfluidizer

Microfluidizer is more efficient for the preparation of emulsions with submicron particles than via homogenization (Jafari, He, & Bhandari, 2007b; Lee & Norton, 2013; McClements, 2015). For example, under same pressure condition (6000 psi) microfluidizer could form finer whey protein stabilized emulsion droplets (about 0.2 μm) than High-pressure valve homogenizer (about 1.3 μm) (Lee & Norton, 2013; McClements, 2015). Meanwhile, there was no significant effect of the number of pass through microfluidizer on mean particle size, while droplet size decreased upon increasing the number of pass through high-pressure valve homogenizer (McClements, 2015). Table 2.2 shows the attributes compared with high-pressure valve homogenizer and microfluidizer.

Table 2.2 Comparison of the attributes of high-pressure homogenizer and microfluidizer

| Homogenizer | Type throughput | Energy density (J m^{-3}) | Relative energy efficiency | Minimum droplet size | Sample viscosity |
|---------------------------------|------------------------|--|-----------------------------------|-----------------------------|-------------------------|
| High-pressure valve homogenizer | Continuous | Medium–high 10^6 – 10^8 | High | 0.1 μm | Low to medium |
| Microfluidizer | Continuous | Medium–high 10^6 – 2×10^8 | High | < 0.1 μm | Low to medium |

2.1.3 Food emulsifiers

2.1.3.1 Role of the emulsifier

The emulsifiers in this review could be defined as the surface-active substance, which are able to adsorb on oil droplets surface and keep emulsion stable (McClements & Gumus, 2016). Emulsifier consists of hydrophobic group and hydrophilic group. The hydrophilic group has an electric charge and can remain in the aqueous phase, while the hydrophobic group can remain in contact with the oil. In this way, emulsifier lowers the interfacial tension between water and oil and stabilizes the oil droplets to avoid phase separation (Jafari, He, & Bhandari, 2007a).

Two key functions of food emulsifiers are listed below (Garti, 1999; Jafari, Assadpoor, He, & Bhandari, 2008; Jafari et al., 2007a, 2007b; McClements, 2015; Santana et al., 2013; Thaiphonit & Anprung, 2016):

1. Emulsion formation:

During the emulsion formation, emulsifier could rapidly adsorb on the surface of oil droplets and reduce its interfacial tension. When reducing the interfacial tension between two immiscible phases, they would form a more stable mixture and less free energy is required to form an emulsion. Meanwhile, emulsifiers could form a protective layer to prevent oil droplets coalescence during homogenization.

2. Emulsion stability:

After emulsion formed, the emulsifiers could continuously prevent the droplets from aggregation and coalescence by the electrostatic or steric repulsion force.

The ideal characteristics of emulsifiers are listed below (Dickinson, 1992; McClements, 2015; Ozturk & McClements, 2016):

1. Surface-activity: Emulsifier must consist of sufficient polar and non-polar groups to ensure them adsorb at the oil-water interface.
2. Adsorption kinetics: Emulsifier should adsorb on the surface of droplets rapidly and effectively decrease interfacial tension. Therefore, they could facilitate droplet disruption and avoid recoalescence happened during homogenization.
3. Stabilization: Emulsifier could form an electrostatic coating or a thick steric coating around oil droplets.

The hydrophilic-lipophilic balance (HLB) value could classify the emulsifying capability. HLB represent the balance of the size and strength of hydrophilic and hydrophobic groups in surfactants, ranging from 1 – 20 (Pasquali, Taurozzi, & Bregni, 2008). The surfactants with high HLB (8-18) have high water solubility and are suitable for O/W emulsions (Kralova & Sjöblom, 2009). However, W/O emulsion formation could be promoted by the surfactants at low HLB (3-6).

The type and concentration of emulsifier are the two crucial factors that determine the size distribution of droplets and emulsion stability. A wide range of emulsifiers is used in food formulations which include, small-molecule surfactants, amphiphilic biopolymers and phospholipids (Krog & Sparso, 2004). For example, different emulsifiers such as modified starch, whey protein and gum Arabic have been used to form emulsions with different particle size (Charoen et al., 2011).

2.1.3.2 Different types of emulsifiers

In the food industry, there are a large amount of synthetic and natural emulsifiers, including Small-molecule surfactants, phospholipids, proteins and polysaccharides (Kralova & Sjöblom, 2009).

Small-molecule surfactants

Small-molecule surfactants are chemical compounds with small molecular weights and high surface-active properties due to their unique structure. These molecules consist of hydrophilic head group and a hydrophobic tail group which have high affinity for water and oil respectively (McClements, 2015; Walstra, 2002). These surfactants could be classified according to their head group into non-ionic, zwitterionic, cationic and anionic. Spans and tweens are non-ionic surfactants, whereas fatty acid salts are anionic and lauric arginate is cationic surfactants. Small-molecule surfactants comparatively have a better efficiency in the stabilization of emulsion than biopolymers, due to their ability to adsorb rapidly at the interface (Jafari et al., 2007a; McClements, 2015).

Phospholipids

Phospholipids or “lecithins” are zwitterionic surfactants that generally act as a major component of cell membranes and in interfacial membranes of naturally occurring oil droplets in plants. The phospholipids are widely used as food emulsifiers and are generally extracted from natural sources rich in oil/fat, such as milk, egg and soybeans (Flanagan & Singh, 2006; Whitehurst, 2008).

The structure of phospholipids consists of two hydrophobic fatty acid “tails” and a hydrophilic “head”, which contains a phosphate group. These two components are assembled together on a glycerol backbone. The unique distribution of hydrophobic and hydrophilic groups on the phospholipid molecule imparts a partial polar character and is responsible for the emulsifying properties of the phospholipids (Contarini & Povolò, 2013). The different phospholipids found in nature can be differentiated based on the different phosphorylated alcohol groups in the polar hydrophilic head. The four most common types of phospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) and their structure is shown in Figure 2.4.

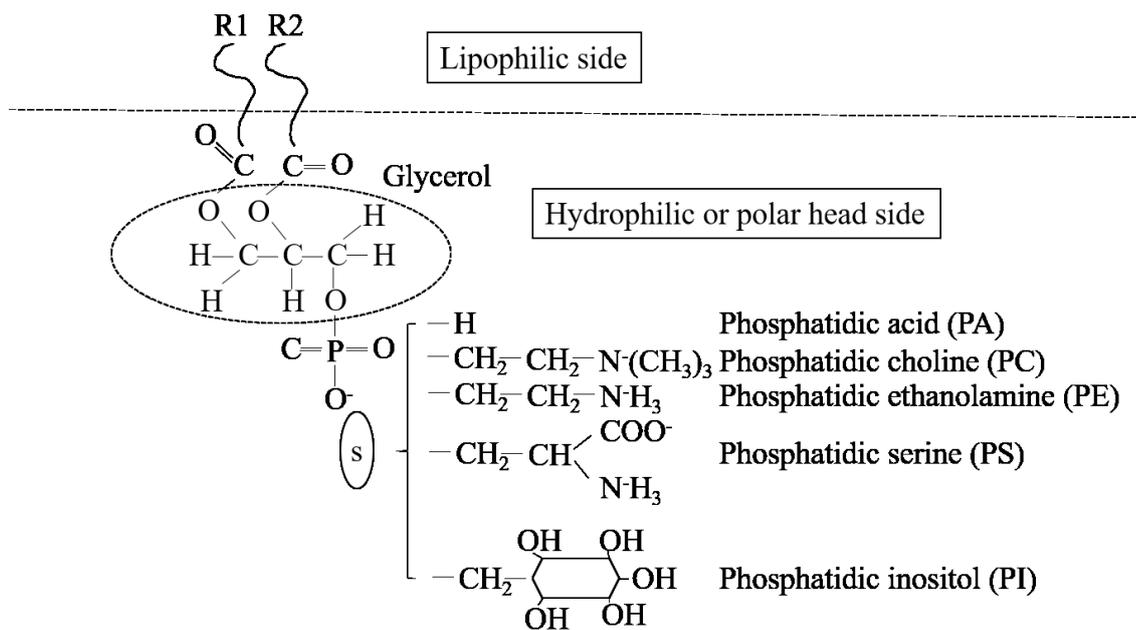


Figure 2.4 Most common types of phospholipids found in nature

In emulsions, phospholipids could form a single interfacial layer on the emulsion oil droplets, when the hydrophobic tails of phospholipids could insert into emulsion oil droplets and hydrophilic heads are toward to water. Alternatively, the phospholipids can also form multiple layers, when their arrangement in the multilayer is tail to tail or head to head (Pichot, Watson, & Norton, 2013). In their natural condition, the HLB number of many phospholipids are around seven, which indicates that phospholipids could be dispersed in water and oil phase before homogenization (McClements, Bai, & Chung, 2017).

The types of phospholipids impact the concentration of the phospholipid required, the structure of the resulting emulsion and its stability (Liu, Ye, & Singh, 2015). Phospholipid-stabilized emulsions are mainly stabilized by electrostatic repulsion, which originates from the electrical charge on phospholipids. At neutral pH conditions, phospholipids stabilized droplets have relatively high negative charge (Ozturk, Argin, Ozilgen, & McClements, 2014). Some phospholipids are considered to have unsatisfactory/poor emulsifying property when used singly due to their low or intermediate HLB numbers. Nevertheless, the combination of phospholipids with other natural emulsifiers, such as whey protein and caseins, has been investigated in recent

years, due to the improvement of emulsifying efficiency, their health benefits and due to their image as “natural” emulsifiers for use in “clean label products” (García-Moreno, Horn, & Jacobsen, 2014).

Biopolymers

Due to the increasing demand for more healthy food, there is a growing interest in using natural ingredients, such as natural biopolymers, to replace the synthetic ingredients (Garti, 1999). Biopolymers contain non-polar groups, which can adsorb at the interface of emulsion droplets surface, and polar groups that spread out in the aqueous phase. Biopolymers exhibit significant surface activity due to their unique polar and non-polar groups (Dickinson, 2003).

Polysaccharides and protein are the most common natural biopolymers that used as food emulsifiers. Proteins adsorb at the interface of the oil and water forming a thin interfacial layer and assist in the formation of an emulsion. The interfacial proteins stabilize the emulsion and prevent coalescence by providing electrical charge and steric interactions (McClements, 2015). When the pH close to isoelectric point or increasing the ionic strength, protein stabilized emulsions tend to aggregate, due to the charge lost and electrostatic screening effects, respectively (Ozturk & McClements, 2016). Polysaccharides, the polymers of monosaccharides, are relatively large molecules and contain relatively less charge than protein. Therefore, they could form a relatively thick coating to protect emulsion droplets by strong steric repulsion (Dickinson, 2003; McClements, 2015).

Proteins from animal-based sources are the most widely investigated (Table 2.3). For example, relatively small droplet ($d_{3,2} = 0.14 \mu\text{m}$) was formed by β -lactoglobulin and lactoferrin (Mao & McClements, 2011). More research on the identification, extraction and application of plant-based sources protein, have been conducted in recent years (Wilde, 2009). For example, soy, pea and lupin proteins could form emulsions, which $d_{3,2}$ are less than $0.4 \mu\text{m}$ (4 %,w/w, oil) (Benjamin, Silcock, Beauchamp, Buettner, & Everett, 2014).

Table 2.3 Studies of proteins as food emulsifiers

| Type of proteins | Concentration of proteins (% w/w) | Concentration of oil (% w/w) | References |
|--|-----------------------------------|------------------------------|------------------------------|
| Whey | 2 | 20 | Hunt and Dalgleish (1995) |
| Caseinates | 1 | 20 | Agboola and Dalgleish (1996) |
| β -lactoglobulin | 0.5 | 10 | Mao and McClements (2011) |
| Lactoferrin | 3 | 10 | Mao and McClements (2011) |
| Lactoferrin and β -lactoglobulin | 0.3 - 3 | 30 | Ye and Singh (2006) |
| Plant protein (of soy, pea and lupin proteins) | 0.31 | 4 | Benjamin et al. (2014) |

Some biopolymer-stabilized emulsions had better stability than phospholipids-stabilized emulsions. Some studies suggest that the combination of biopolymer and phospholipids could act as good emulsifiers (McClements & Gumus, 2016). For instance, caseins mixed with lecithin was investigated to stabilize antimicrobial emulsions (Xue & Zhong, 2014).

2.1.4 Emulsion characterization

2.1.4.1 Particle size distribution

The particle size of emulsion would determine many properties of the emulsion, including the appearance, stability and texture. Most emulsions are polydisperse with droplet size, from 100 nm to 100 μ m (Dickinson, 1992). Hence, it is common to express the droplet size in a size range rather than by a single number. The range could represent the distribution of particle size with respect to the volume, number or surface area of particles. However, the mean particle size is also commonly used to describe the emulsion droplet size (Dickinson, 2003). The $d_{3,2}$ and $d_{4,3}$ are the two most common ways to interpret the mean droplet size. The $d_{3,2}$ refers to the average surface area of droplets in the emulsion, while the $d_{4,3}$ represents the average volume of droplets in the emulsion (McClements, 2015). The $d_{3,2}$ is less sensitive to the emulsion which contains large particles than $d_{4,3}$,

therefore, it is preferred to explain the aggregation phenomena e.g. flocculation (Dickinson, 2003).

2.1.4.2 Droplet charge

The droplets in the emulsion are coated by emulsifiers and the surface charge is mainly decided by the properties of the emulsifiers, since the hydrophilic head of emulsifiers may be negatively, positively or neutrally charged. The surface charge on droplets plays an important role in generating electrostatic repulsion to prevent droplet coalescence. The ζ -potential is usually used to denote the droplet surface charge and could be measured by dynamic light scattering. An electrical field is applied to the emulsion in a cuvette, which has a pair of electrodes. The emulsion droplets in cuvette move to the oppositely charged electrode depending on their charge. The ζ - potential would be calculated based on the moving velocity, size and shape (Hunter, 2013).

2.1.4.3 Microstructure

Although particle size analysis could provide droplet size information, the process of sample preparation such as dilution or agitation may affect the microstructure of emulsion. The use of microscopy to understand the microstructure of emulsion serves as an important technique to study emulsion structure without affecting its properties. Generally, optical microscopy, confocal laser scanning microscopy (CLSM), electron microscopy are commonly used for different test requirements and emulsion types (McClements, 2015). Compared with optical microscopy, CLSM can produce images with higher resolution and clarity. CLSM is a good method for investigating emulsion structure by using fluorescent dyes which could selectively bind to specific target components, such as fat (polar as well as non-polar), protein (with and without glycosylation) and carbohydrates. Therefore, through the CLSM image, the structure of emulsion could be detected, such as droplet size, emulsion aggregation state, distribution of interfacial components (fat, protein, phospholipids and polysaccharides) (McClements, 2015).

2.1.5 Emulsion stability

All emulsions are thermodynamically unstable and tend to separate into oil and water phase. However by using emulsifiers and adjusting homogenization condition and

process could increase the kinetic stabilities of emulsions (Jafari et al., 2008). There are several mechanisms that could explain emulsion destabilization, such as creaming, flocculation and coalescence (Dickinson, 1992; McClements, 2015). Figure 2.5 shows the three types of physical destabilization of the emulsions.

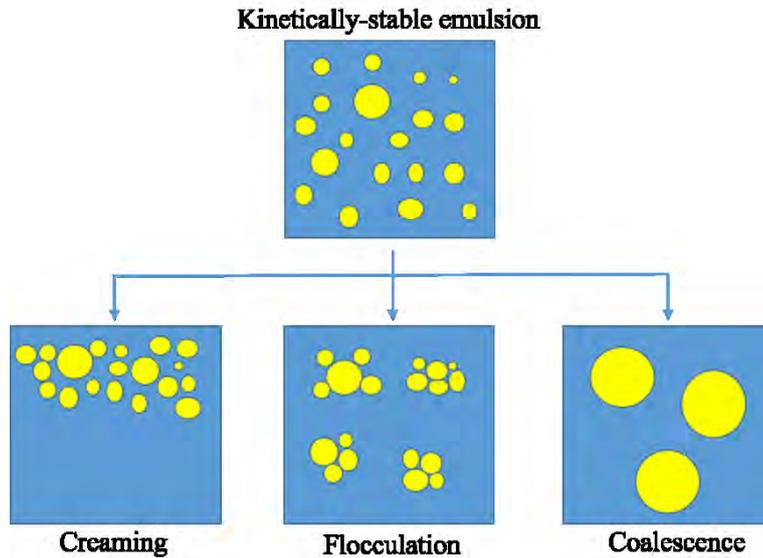


Figure 2.5 Physical changes in O/W emulsion. The yellow circles represent oil droplets while the blue shaded area represents continuous phase

2.1.5.1 Creaming

Creaming refers to the separation of oil droplets, in the emulsion held under the force of gravity into a droplet-rich phase on top. The droplets move upward and form a cream layer on the top of the emulsion without any change in the droplets size distribution (Figure 2.5) (Dickinson, 1992). The rate of creaming could follow the Newtonian fluid is given by Stoke's law:

$$v = \frac{2gr^2(\rho_2 - \rho_1)}{9\eta} \quad (\text{Eq. 2.1})$$

where v is the velocity of creaming, g is the acceleration of gravity, r is the droplet radius, ρ_1 and ρ_2 are the density of oil phase and aqueous phase respectively, η is the viscosity of aqueous phase (McClements, 2015).

According to the equation, in a given matrix, the difference of densities of oil and aqueous phases is one of the major reasons responsible for the creaming. The creaming rate could be reduced by decreasing the droplet size and oil density. The thickness of the cream layer depends on the effectiveness of packing. Tightly packed droplets may generate thinner cream layer than their loosely packed counterparts. The creamed emulsion can usually be dispersed by mild agitation and can be determined by measuring the height of the cream layer over time (McClements, 2015).

2.1.5.2 Flocculation

Flocculation is a reversible aggregation phenomenon when emulsion droplets form loose aggregates but they still keep their individual integrity. The emulsion droplets are forced by the shear flow and are in continuous Brownian motion, resulting in collisions with each other. During these collisions, depending on the intensity of attractive and repulsive forces between the droplets, either associate together or separate again (Dickinson, 1992). The association of droplets upon collisions results in flocculation. The flocculation of droplets can occur via two different mechanisms: Bridging flocculation or depletion flocculation. Bridging flocculation occurs when there are not enough biopolymer emulsifiers to cover all the droplets surface area, and two or more droplets may share one single emulsifier molecule (Dickinson, 1992). Depletion flocculation arises when emulsion droplets are close to each other and the polymer molecules are excluded from the area between them (McClements, 2015).

The rate of flocculation is mainly dependent on the interfacial layer, which consists of emulsifiers. In a stable emulsion, droplets could be prevented from flocculation either by electrostatic or steric stabilization. Electrostatically stabilized droplets keep individual integrity by electrostatic repulsion which is contributed by charged emulsifiers, such as proteins (Damodaran, 1994). For example, β -lactoglobulin was stable at pH7, which ζ -potential was at 60 mV (Mao & McClements, 2011). In sterically stabilized emulsions, biopolymer emulsifiers are fully cover on the surface of droplets and create thick interfacial membrane the protect droplets (McClements, 2015). For instance, emulsions stabilized by gum Arabic were stable at pH 3 to 9 (Chanamai & McClements, 2002). The degree of flocculation can be measured by measuring the size of emulsion droplets and comparing it with that when the flocks are dispersed by changing emulsion conditions.

This can be brought about by changing the ionic strength, pH or by using small-molecule surfactants to desorb the biopolymers at the interface (McClements, 2015).

2.1.5.3 Coalescence

Unlike flocculation, coalescence is an irreversible aggregation phenomenon which occurs due to the rupture of the interfacial membrane and merger of two or more small droplets into a large droplet (Figure 2.5). There are two mechanisms that can explain coalescence. Firstly, like flocculation, coalescence can occur due to the collision. The rate of this coalescence is depended on the same factors as flocculation mentioned above. Secondly, if creaming or flocculation occurs, coalescence may follow if the contact times between the droplets is sufficiently long. Hence, the properties of the interfacial layer could strongly decide the coalescence of emulsion (Hunter, 2013). The coalescence of emulsion droplets can be prevented by selecting emulsifiers that provide high electrostatic repulsion and steric hindrance. Some proteins were proved that they could provide both two repulsions (McClements, 2015). The coalescence in emulsions can be known by measurement of droplet sizes by viewing them under a microscope or by particle size measurement in the presence of a small-molecule surfactant (Demetriades, Coupland, & McClements, 1997).

2.1.6 Factors affecting emulsion stability

During the processing and storage of emulsion, a wide range of factors would affect the emulsion stability. These factors could be pH, ionic strength, thermal treatment, etc.

2.1.6.1 pH

The pH of continuous phase has a major effect on the surface charge of the emulsion droplets, which in turn would significantly influence emulsion stability. As the pH approaches the pI, the negative or positive charges on the droplet surface are screened and the ζ - potential value becomes close to 0. In the absence of the charges, the emulsion tends to flocculation, since the electrostatic repulsion is not strong enough to counter the attractive force between droplets. On the contrary, when the pH is raised away from the emulsion pI, the droplet surface is highly charged, which could produce strong electrostatic repulsion to prevent from coalescence and flocculation (McClements, 2015).

2.1.6.2 Ionic strength

The salts present in the emulsion can screen the charge on the droplet surface and binding the oppositely charged groups to reduce the ζ - potential value. Subsequently, the electrostatic repulsion would decrease and flocculation would be induced (Guzey, Kim, & McClements, 2004; McClements, 2015). Monovalent cations, Na^+ and K^+ from NaCl and KCl, and divalent cations, Ca^{2+} from CaCl_2 are usually be introduced in the emulsion to investigate emulsion stability against ionic strength change. Some researches pointed out that adding NaCl (≥ 150 mM) could lead to significant flocculation and creaming in WPI stabilized emulsion at pH 3 (Djordjevic, Kim, McClements, & Decker, 2004). However, in case of sodium caseinate stabilized emulsion, α_{s1} -casein are more susceptible to flocculation than β -casein, which showed no aggregation, at the same salt condition (100 mM NaCl) (Dickinson, 1997). It indicated that the influence of ionic strength on emulsion stability may also be affected by the types of protein adsorb on the surface of droplets.

2.2 Oil bodies in plants

2.2.1 Structure of oil bodies

Oil bodies present as discrete organelles and mainly located in plant seeds, nuts and some fruits (Nikiforidis et al., 2014). Their primary function is to storage triglycerides (Tzen, Peng, Cheng, Chen, & Chiu, 1997). Generally, the size of oil bodies is 0.5-2.0 μm in diameter in seeds and 10-20 μm in diameter in fruits (Ross, Sanchez, Millan, & Murphy, 1993). The oil bodies in natural condition are remarkably stable, due to the electrostatic and steric repulsion provided by interfacial (Nikiforidis et al., 2014). The interests of oil bodies were raised due to their potential applications in the delivery of bioactive compounds (Acevedo et al., 2014). The structure of oil body consists of a triglycerides core (95%, w/w) covered by an interfacial layer, which consist of phospholipids monolayer (up to 2% w/w) and oil body membrane proteins (oleosins) (up to 4%), shown in Figure 2.6 (Huang, 1996; Napier, Stobart, & Shewry, 1996; Yamaguchi, 2010). The four most common types of phospholipids were phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) (Yeagle, 2016).

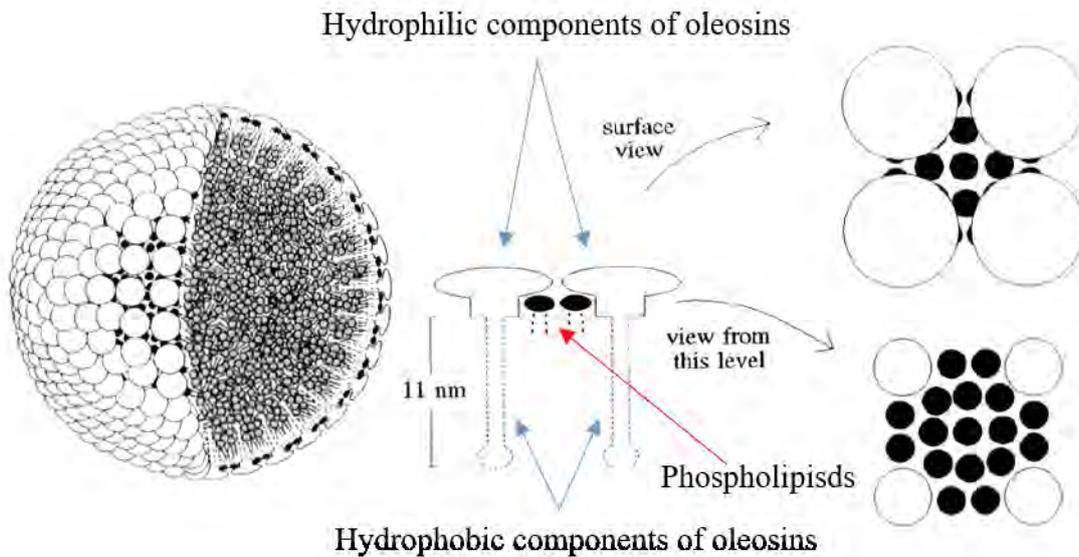


Figure 2.6 The model of seed oil body. Reproduced with permission from Tzen and Huang (1992)

Oil bodies have some unique proteins at the interface, which are called oleosins. They are a group of proteins with molecular weights ranging from 15 to 30 kDa (Tzen & Huang, 1992; Yamaguchi, 2010). Nikiforidis et al. (2014) noted that the entire oil body surface may be covered by oleosins. The negative charge carried by proteins in natural state causes electrostatic repulsion resulting into stabilization of oil bodies (Yamaguchi, 2010). In addition, the proteins also stabilize the oil body structure by steric interactions.

The primary structure of oleosins consists of the hydrophilic C-terminal domain, hydrophilic NH₂-terminal domain and hydrophobic central domain (which insert an anchor in the triglyceride core and enable oleosin inserts into oil bodies) (Figure 2.7). The N and C terminal domains lie on and cover the oil body surface, while the central domain acts as an anchor and embeds in the matrix of triacylglycerol core and hydrophobic moieties of phospholipids (Napier et al., 1996; Nikiforidis et al., 2014; Tzen & Huang, 1992). Oil bodies also consist of a monolayer of phospholipids with embedded proteins (Boucher, Cengelli, Trumbic, & Marison, 2008). Huang (1996) pointed the phospholipids layer surround the triglyceride core and shield by unique oleosin layer. The phospholipids coat also prevent oil bodies from aggregation and coalescence.

The oil body size is highly correlated with the triglycerides to oleosin ratio. Generally, higher triglycerides to oleosin ratio might cause large oil body size and vice-versa. For example, avocado has high triglycerides to oleosin ratio and relatively large oil body size (20 μm in diameter) (Platt-Aloia & Thomson, 1981).

2.2.2 Oil bodies extract materials as food emulsifiers

Since the oil bodies interfacial membrane shows the great ability of against coalescence, people suggested that oleosin and phospholipids could perform as ideal emulsifier materials. The applications of reconstituted oil body were investigated, such as serve as bio-capsule to encapsulate the lactic acid bacteria (Hou, Lin, Wang, & Tzen, 2003) and hydrophobic molecules (like nutraceutical compounds) carrier (Chen, Chyan, Lee, Huang, & Tzen, 2004). Many researchers has successfully extracted oil body interfacial membrane from different plant oil bodies (Chen et al., 2004; Peng, Lin, Lin, & Tzen, 2003; Ross et al., 1993; Tzen & Huang, 1992; Tzen et al., 1997; White et al., 2008). Generally, there are two steps to obtain the oil bodies extracts materials. The first step is oil body isolation (oil body based emulsion or cream) (Rosenthal, Pyle, & Niranjana, 1996). Aqueous media extraction is used instead of conventional organic solvent extraction. The advantage of aqueous extraction is that it keeps the oil body intact released into aqueous media. Therefore, both endogenous protein (on the oil body surface) and storage protein (anchor in the triglyceride core) would be extracted simultaneously (Nikiforidis et al., 2014). The second step is demulsification (Rosenthal et al., 1996). At this step, many different methods were used to release oil body interfacial material. Tzen and Huang (1992) used organic solvent extraction (diethyl ether, chloroform and methanol) to separate proteins, phospholipids and neutral lipids. Onsaard, Vittayanont, Srigan, and McClements (2006) used isoelectric precipitation to obtain oil body protein. Gunetileke and Laurentius (1974) and Onsaard et al. (2006) used freeze-thaw treatment to obtain oil body interfacial materials.

The difference of the membrane structure of natural oil body and reconstituted oil body could be investigated by using different enzymes to disturb the membrane of emulsion droplets. Pepsin has highest efficiency in cleaving aromatic amino acids and hydrophobic residues in protein (Fruton, 2002). Trypsin predominantly cleaves the positively charged lysine or arginine amino acids at C-terminal (Ma, Tang, & Lai, 2005). Phospholipase A₂ can act on carboxylic esters at sn-2 of phospholipids and digest phospholipids to free fatty

acids and lysophospholipids (Nik, Wright, & Corredig, 2010). Tzen and Huang (1992) used trypsin and phospholipases to treat both natural maize oil body and its reconstituted oil body formed by their constituents (triglyceride, phospholipids and proteins). Trypsin induced the natural oil body coalescence but phospholipases did not, while phospholipases showed enzymatic activity on trypsin treated (30 min) oil body. Same phenomena were observed that the reconstituted oil body (with the native triglyceride, phospholipids and proteins proportion, 97.7 %, 0.9 % and 1.4%, w/w) was not stable under trypsin treatment but not phospholipases treatment. This result suggested reconstituted maize oil body might have similar membrane structure with the native one. They also suggested that the rapeseed, rice, wheat and soybean reconstituted oil body (formed at the same constituents ratio as maize oil body) were stable without enzymatic treatment.

2.3 Coconuts

Coconut (*Cocos nucifera* L.) is one of the most well-known monocotyledon palms cultivated because of its significant economic value (Green, 1991; Heathcock & Chapman, 1983; Seow & Gwee, 1997; Solangi & Iqbal, 2011). More than 97% of coconut production is contributed by the countries from Asia, the Pacific islands and Central and South America. Among them, India, Indonesia and Philippines are the 3 largest coconut producers, which can produce about 3.5 million MT, 3.2 million MT and 2.7 million MT in copra equivalent (Amrizal, 2003). The coconut fruits are widely used for the extraction of coconut oil due to its economic value. Besides coconut oil has a number of physicochemical properties that are beneficial for food processing and has health benefits for consumers. Coconut oil is clear liquid at 40 °C oil and sweet characteristic flavour (Bhatnagar, Prasanth Kumar, Hemavathy, & Gopala Krishna, 2009) and has a melting point of 25 to 28 °C (Granger, Schöppe, Leger, Barey, & Cansell, 2005). Most of the triglycerides of coconut oil contain saturated fatty acids (92.7%) and the unsaturated fatty acids account for <5%, of the total fatty acids with oleic and linolenic acids being the main unsaturated fatty acids (Appaiah, Sunil, Kumar, & Krishna, 2015; Appaiah, Sunil, Prasanth Kumar, & Gopala Krishna, 2014; Bhatnagar et al., 2009).

2.3.1 Structure of coconut fruit

The structure of coconut fruit is shown in Figure 2.7. Mature coconut fruit consists of 6 distinct regions which are exocarp, mesocarp, endocarp, kernel, testa and water. The fruit consists of fibrous tissues of exocarp and mesocarp which are generally removed at maturity before commercial sale. The commercially sold coconut consists of the endocarp: an outer hard shell with thickness up to 0.4 to 0.5 mm, the testa: a brown layer (0.2 mm) that separates the endocarp from the rest of the fruit and the endosperm regions (solid and liquid). The endosperm regions of the coconut form the edible parts of coconut and have been used for the production of different coconut food products.

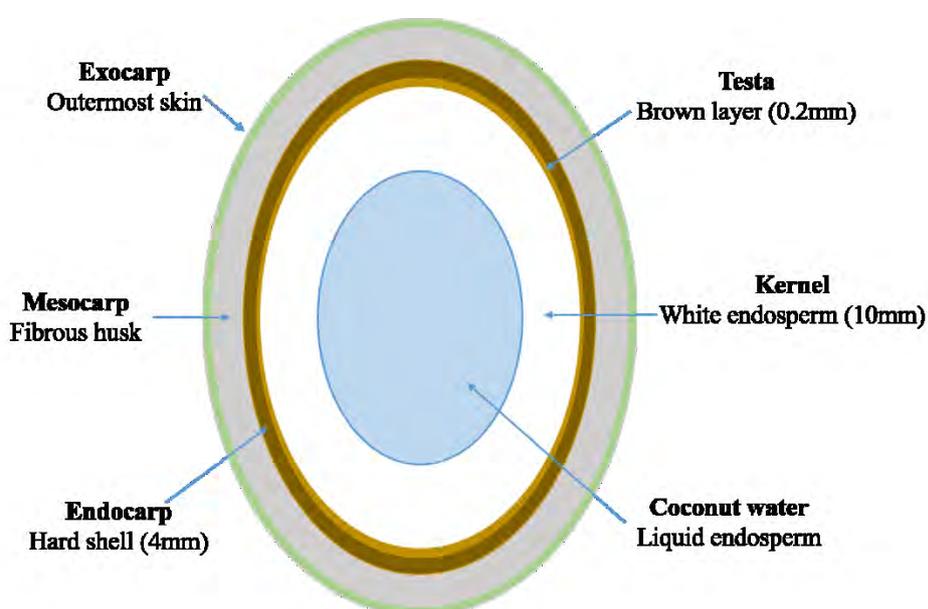


Figure 2.7 Parts of mature coconut

The liquid endosperm of coconut or coconut water is devoid of any oil and is present in the hollow cavity of the coconut. It is low in carbohydrates but high in minerals (Grimwood & Ashman, 1975; Seow & Gwee, 1997). In comparison, the solid endosperm also known as “coconut meat” or “coconut kernel” contains all of the coconut oil along with proteins some sugars and dietary fibre, and forms the raw material for the extraction and manufacture coconut milk and cream (Heathcock & Chapman, 1983; Tangsuphoom & Coupland, 2008). This oil is mainly contained as spherical droplets embedded in the cells called oil bodies. The oil bodies from coconuts have been discussed in detail in Section 2.3.4. The typical composition of coconut kernel and testa (brown skin layer covered kernel) is shown in Table 2.4. Appaiah et al. (2014) report that the proximate

composition, especially in the fat and protein content of the kernel was similar to the testa material removed during parring in an industrial set-up. The coconut kernel is used for the preparation of a number of coconut products such as coconut milk, coconut cream, desiccated coconut powder and grated coconut.

Table 2.4 The composition of coconut kernel and testa (Appaiah et al., 2014)

| Sample | Content (%) | | | | | |
|----------------|-------------|------|---------|---------------|-------------|-----|
| | Moisture | fat | Protein | Carbohydrates | Crude fibre | Ash |
| Coconut kernel | 43.5 | 38.8 | 6.2 | 10.6 | 11.7 | 0.9 |
| Coconut testa | 32.9 | 34.7 | 7.1 | 24.6 | 17.2 | 0.7 |

2.3.2 Coconut milk

Pressing the solid endosperm expresses a white milky liquid extract which is rich in fat. This aqueous extract is referred to “coconut milk” which has a pH of 6.2 (Seow & Gwee, 1997; Tangsuphoom & Coupland, 2008). The Malaysian food regulations defined coconut milk to contain a minimum of 30% of fat and 3% of protein and less than 55% of water. Coconut cream, which is collected by coconut milk separation process, should have more than 50% of fat and 5% of protein (Seow & Gwee, 1997). The Codex Standard for Aqueous Coconut Products (CODEX STAN 240-2003) defined that the minimum amount of fat and non-fat solids are 10% and 2.7%, respectively (Tangsuphoom & Coupland, 2008).

The steps involved in the extraction of coconut milk are shown in Figure 2.8 (*Coconut Handbook*, 2016). The extraction yield and composition of coconut milk are decided by many factors, such as extraction pressure, kernel: water ratio, coconut maturity and breed, size of coconut kernel mince (Cancel, 1970; Waisundara, Perera, & Barlow, 2007). Cancel (1970) reported that there was no significant impact on coconut milk composition by using different water to coconut kernel ratio (0:4, 1:4, 2:2, 3:4, 4:4) and water temperature (27-30°C, 88-93°C).

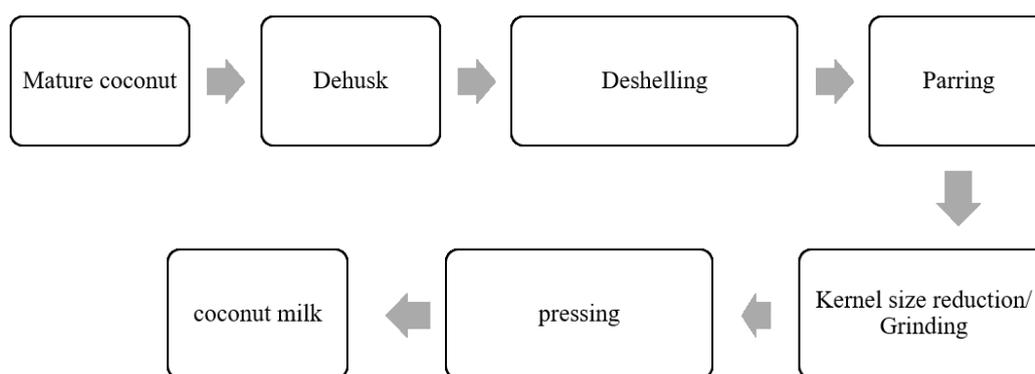


Figure 2.8 Steps involved in the manufacture of coconut milk

2.3.3 Coconut milk composition and properties

The composition of coconut milk is dependent on the amount of water used for extraction. Typical composition of freshly extracted coconut milk is shown in Table 2.5. The majority of minerals in coconut milk is phosphorous, potassium, calcium.

Table 2.5 Proximate composition of the kernel from fresh mature coconut (Ohler, 1999; Seow & Gwee, 1997; Tangsuphoom & Coupland, 2008)

| Sample | Content (%) (wet weight basis) | | | | |
|--------------------------------------|--------------------------------|-------|---------|---------------|-------|
| | Moisture | Fat | Protein | Carbohydrates | Ash* |
| Fresh coconut endosperm | 44-52 | 35-38 | 3-4 | 9-11 | 1 |
| Coconut milk extracted without water | 54.1 | 32.2 | 4.4 | 8.3 | 1 |
| Coconut milk extracted with water | 73-76 | 19-21 | 2-3 | 1-1.6 | 0.6-1 |

* The majority of minerals in coconut milk is phosphorous, potassium, calcium (Seow & Gwee, 1997).

2.3.3.1 Coconut fat

1) Triacylglycerol

Coconut oil is clear oil which is extracted from coconut kernel and milk and contains sweet and special flavour (Bhatnagar et al., 2009). The melting point of coconut is at the

range of 25-28°C (O'Brien, 2008). Coconut lipids are made up of 94.3% of neutral lipids (Krishnamurthy & Chandrasekhara, 1983). Meanwhile, previous studies have also pointed out that there are 84% (w/w) of triglyceride, 12% (w/w) of diglyceride and 4% (w/w) of monoglyceride in coconut fat (O'Brien, 2008; Relkin, Fabre, & Guichard, 2004). The physical properties of coconut oils are determined by the composition of coconut triglycerides. Most of triglycerides of coconut oil consist of saturated fatty acids (92.7%, w/w) which results in a high stability to oxidation, while the unsaturated fatty acids constitute to less than 5% (w/w) of total fatty acids in coconut oil (Appaiah et al., 2014; Bhatnagar et al., 2009). The medium-chained fatty acids (C6-C14) form 80% of the total fatty acids of coconut oil and are also responsible for the health benefits associated with the consumption of coconut oil. Medium chain fatty acids are transported to the liver for their breakdown for energy production. Therefore, less accumulation of fat in human body, which in turn reduces the risk of occurrence of cardiovascular diseases.

Table 2.6 Fatty acid composition of coconut oil (Bhatnagar et al., 2009)

| Fatty acid | %, w/w |
|-----------------------------|---------------|
| C8:0 | 5.8 ± 0.4 |
| C10:0 | 4.8 ± 0.3 |
| C12:0 | 49.1 ± 1.6 |
| C14:0 | 21.8 ± 1.1 |
| C16:0 | 8.4 ± 0.8 |
| C18:0 | 2.8 ± 0.2 |
| C18:1 | 6.1 ± 0.3 |
| C18:2 | 1.2 ± 0.2 |
| Saturated Fatty Acids | 92.7 ± 4.4 |
| Monounsaturated Fatty Acids | 6.1 ± 0.3 |
| Polyunsaturated Fatty Acids | 1.2 ± 0.2 |
| Medium-Chain Fatty Acids | 59.7 ± 2.3 |

The details of fatty acid composition of coconut oil, their distribution in triacylglycerol of coconut fat and their health benefits have been discussed in previous reviews (Appaiah et al., 2015; Appaiah et al., 2014; Bhatnagar et al., 2009; Cancel, 1970; *Coconut*

Handbook, 2016; Krishnamurthy & Chandrasekhara, 1983; O'Brien, 2008; Relkin et al., 2004).

2) Minor lipids

The coconut fat contains approximately 5.5% (w/w, total fat) of glycolipids and 0.2% (w/w, total fat) phospholipids. The phospholipid composition of coconut fat is shown in (Table 2.7) (Krishnamurthy & Chandrasekhara, 1983). The fatty acid composition of these lipids is not known while they are expected to be present at the interfacial layer surrounding the coconut triacylglycerols in the oil bodies.

Table 2.7 Different types of phospholipid in coconuts

| Phospholipids | % of total phospholipids (w/w) |
|-------------------------------|-----------------------------------|
| Phosphatidyl choline | 34.6 |
| Phosphatidyl ethanolamine | 24.6 |
| Phosphatidyl inositols | 19 |
| Phosphatidyl serine | 4.8 |
| Lysophosphatidyl choline | 4.6 |
| lysophosphatidyl ethanolamine | 3.4 |
| Phosphatidic acid | 2.5 |

2.3.3.2 Coconut proteins

There are approximately 5-10% (w/w, weight on dry basis) of protein in coconut milk (Seow & Gwee, 1997). Balachandran and Arumugan (1992) and Rasyid, Manullang, and Hansen (1992) reported that globulins (75% of total protein, which 86% of 11S globulins and 14% of 7S globulins) and albumins (rest 25%) are the main protein species in coconut endosperm and most of them also present in coconut milk. Most of the coconut milk proteins are retained in the coconut skimmed milk upon centrifugation. Hagenmaier, Cater, and Mattil (1972) reported that only around 30% of the total proteins present in coconut milk to be present in filtered coconut milk aqueous phase and proposed that the rest of the undissolved proteins in aqueous could act as emulsifying agent for oil bodies or be associated with the oil bodies.

When analyzed by SDS-PAGE under non-reducing conditions, coconut milk proteins, included six protein bands, which molecular weight were 55 (11S globulins), 46, 33, 25, 18 and 16 kDa. Disulphide bridge links 32-34 kDa (acidic) and (22-24 kDa (basic) subunits to form the 55 kDa protein (Benjakul, Patil, Prodpran, Senphan, & Cheetangdee, 2016; Garcia, Arocena, Laurena, & Tecson-Mendoza, 2005). Under the reducing condition, the molecular weight of 55, 33, 31, 25, 21, 20, 18 and 16 kDa were observed (Benjakul et al., 2016). Kwon, Park, and Rhee (1996) pointed out that the different types of coconut proteins (globulin and albumin) may link together through disulfide bonds. The bands of globulin could be detached in reducing condition, while the most bands of albumin persisted in reducing condition, which means there were relatively low disulfide bridge in albumin. These results also be confirmed by Garcia et al. (2005) and Tangsuphoom and Coupland (2009).

The amino acid composition of coconut proteins is listed in Table 2.8. Glutamic acid (24%), arginine (13.8%), and aspartic acid (9.5%) constitute the majority of charged residues in coconut milk proteins (Rasyid et al., 1992). The isoelectric point of coconut milk proteins lies between pH 4 and 5, while their solubility increases with increase in pH reaching a maximum in solubility between the pH 6 to 8. The natural pH at extraction of coconut milk is noted to be 6.2 (Onsaard, Vittayanont, Srigam, & McClements, 2005).

Table 2.8 Amino acid composition of coconut protein

| Amino acid | % (W/W) |
|---------------|---------|
| Alanine | 8.0 |
| Arginine | 13.8 |
| Aspartic acid | 9.5 |
| Cysteine | 0.4 |
| Glutamic acid | 24.0 |
| Glycine | 7.3 |
| Histidine | 1.7 |
| Isoleucine | 3.3 |
| Leucine | 6.0 |
| Lysine | 4.7 |
| Methionine | 0.2 |
| Phenylalanine | 3.5 |
| Proline | 4.5 |
| Serine | 3.4 |
| Threonine | 2.9 |
| Tyrosine | 1.0 |
| Valine | 6.0 |

2.3.4 Coconut oil body

In their natural state, the coconut triacylglycerol is contained as oil bodies within the cells of the kernel tissues. Heathcock and Chapman (1983) investigated the distribution of oil-rich cells in the kernel and noted that the distribution of cells rich in oil bodies varies significantly in the kernel. There is a large amount of oil accumulated in the outer and middle endosperm cells.

In a freshly extracted suspension, the coconut oil bodies appear spherical and their size ranges from 2 to 25 μm in diameter (Tangsuphoom & Coupland, 2008). At natural pH (6.2) of coconut milk extraction, the net charge on the oil body is -16 mV, which is mainly contributed by the interfacial proteins on the oil body surface (Kwon et al., 1996; Samson, Khaund, Cater, & Mattil, 1971; Tangsuphoom & Coupland, 2009). Freshly extracted coconut milk when allowed left unstirred, would spontaneously separate into a top oil body-rich layer, a clear protein rich middle layer and a bottom insoluble sediment layer.

Recent research has shown that the freshly extracted oil bodies had a polydisperse size distribution and their $d_{4,3}$ and the $d_{3,2}$ were 15.7 ± 2.1 and 9.5 ± 1.8 μm , respectively. These values did not change ($d_{4,3}$ was 15.5 ± 1.6 and $d_{3,2}$ was 8.8 ± 1.1 μm) with 1% SDS, which indicate that oil bodies in nature condition were free to flocculation. Oil bodies follow the same structural design principles as those of oil bodies from seeds. The interfacial layer of coconut oil bodies consists of phospholipids and proteins (Figure 2.9) (Dave, Ye, & Singh, 2019).

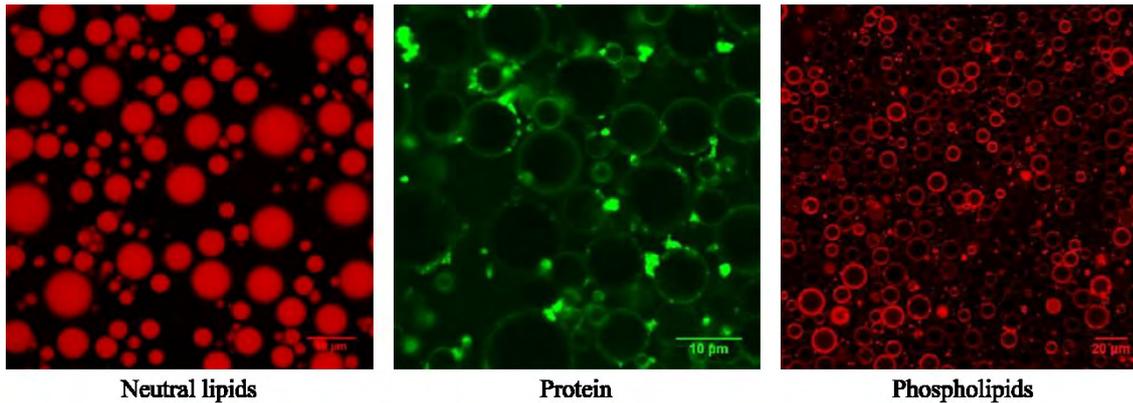


Figure 2.9 Confocal laser scanning microscopy of freshly extracted coconut oil bodies (Dave et al., 2019)

2.3.4 Destabilization of coconut oil emulsion

The structure of oil bodies from plants is similar to that of milk fat globules where the triacylglycerol core is covered by an interfacial layer consisting of proteins and phospholipids layer (Singh, 2006). Therefore, the knowledge of extracting milk fat globular membrane (MFGM) may give us an idea of broken coconut emulsion. For commercial-scale production, to release milk fat globular membrane (MFGM) from fat globule to serum phase, there are 4 main steps, which are cream separation, cream washing, MFGM releasing (freeze-thaw) and MFGM collection (Patton & Keenan, 1975; Singh, 2006). During the freeze-thaw treatment with churning, fat crystal would be formed and break the MFGM at low temperature (Dewettinck et al., 2008).

Coconut milk can be treated as an O/W emulsion which is stabilized by coconut phospholipids and proteins (Dave et al., 2019). To obtain oil body interfacial components as natural emulsifiers, destabilizing emulsion approaches are widely applied. The most widely used method for destabilization of coconut oil body emulsion is the use of higher temperature in the manufacture of coconut oil. Destabilization of coconut oil bodies by

mechanical treatments or without heating has also been used in the manufacture of virgin coconut oil. Different methods including thermal treatment (40-90 °C for 20 min), changing pH (3-10), freeze-thaw treatment (from 5 °C to 30 °C), enzymatic treatment (aspartic protease; 2500 tyrosine units/g; 0.1% concentration; incubated at 25 and 37 °C for 3 h) used singly or in combination have been investigated. Table 2.9 lists the oil yield for each treatment (Raghavendra & Raghavarao, 2010). These authors compared the efficiency of separation of free oil upon centrifugation of samples treated by the above methods.

Table 2.9 The yield of coconut oil from coconut kernel using different treatments (Raghavendra & Raghavarao, 2010).

| Treatment | Condition | oil yield (% , w/w) |
|------------------------------------|---------------------------|----------------------------|
| Thermal treatment | 90 °C | 86% |
| pH treatment | pH 3 | 89% |
| Freeze-thaw treatment | from 5 °C to 30 °C | 92% |
| Enzyme treatment | 37 °C | 83% |
| Enzyme followed chilling treatment | 37 °C; from 5 °C to 30 °C | 95% |

A recent study has shown that the oleosins at the coconut oil body interface have extensive disulfide bonding. Although the possibility of disulfide bonding in interfacial proteins in oil bodies of other plant sources has not yet been considered, its presence in coconut oil body suggests that thermal treatment may cause denaturation and disulfide-linked covalent aggregation of oleosins. High heat-treatment would alter the native-like character of the oleosins and hence emulsifying properties. In comparison, the enzymatic treatments rely on access of the enzyme to the site of action. If there are insufficient cleavage sites or these sites are not accessible to the enzyme, a lower yield of the oil (Table 2.9) and the oil bodies may result. In addition, it is also possible that the protease cleavage is unable to destabilize the oil bodies significantly since the oil bodies have an additional phospholipid layer at the interface. Besides, it is not known what impact does proteolytic cleavage of the oleosins has on their emulsification properties when making an emulsion. Thus, when choosing the extraction method of oil body membrane for use as emulsifying agents, the effect of the extraction conditions on the phospholipids and oleosins should be considered.

2.3.5 Coconut oil body proteins as emulsifying materials

The coconut oil bodies being bigger in size than the seed oil bodies have a larger volume but a significantly smaller surface area. Thus, the yield of interfacial biomaterials is expected to be lower than that from the oil bodies in seeds. Nevertheless, the oleosins and polar lipids at the oil body interface provide an important source of natural emulsifiers and their extraction serves as an important stream from the by-product stream in the manufacture of virgin coconut oil.

Onsaard et al. (2005, 2006) investigated the properties of emulsions stabilized by coconut skimmed milk proteins and cream proteins. The raw materials (coconut skimmed milk and cream) were obtained by using centrifugation. Coconut skimmed milk protein concentrate (CSPC) and isolate (CSPI) prepared by membrane processing and isoelectric precipitation were used in the study. The coconut cream proteins were prepared using two different protocols: Isoelectric precipitation (CCP1) and freeze-thaw treatment (CCP2). Corn oil emulsions with final oil concentration of 10% (w/w) corn oil emulsions (CSPI, CSPC, CCP2) stabilized by 0.2% (w/w) extracted proteins (solution prepared with pH 6.2 buffer) were produced by high-pressure valve homogenizer. These authors noted considerable flocculation of emulsions close to the isoelectric point pH of proteins (pH 4 of CSPI, pH 4.5 of CSPC,). Meanwhile, the CSPI and CSPC could form relatively constant emulsion droplets at 0.2-1.0% (w/w) protein concentrations ($d_{3,2}$ was 0.4-0.5 μm and 1.0-1.5 μm , respectively). CCP2 formed smaller emulsion droplets ($d_{3,2}$ was 2 μm) than CCP1 did ($d_{3,2}$ was 5 μm) at same protein concentrations. CSPI and CCP2 emulsions were relatively stable at low NaCl concentrations (≤ 50 mM) and thermal treatment (at $\leq 80^\circ\text{C}$ and $\leq 90^\circ\text{C}$ for 30 min, respectively). However, the comparison of different protein concentrations for emulsification and the maximum quantity of oil that could be loaded in the emulsions was not considered in their studies. Moreover, these authors considered homogenization as a method for emulsification, probably with an aim to develop food applications. However, it is not known whether smaller droplet sizes could be achieved using these materials which would make them suitable for applications in pharmaceuticals such as encapsulation of bioactive or drug delivery, since a smaller droplet size would increase the surface area and increase bioaccessibility and hence bioavailability of the bioactive/drug.

3. Materials and Methods

3.1 Materials

Unless stated otherwise, all chemicals were purchased from Sigma Aldrich Ltd. (St. Louis, MO, USA) and the reagents were made up in Milli Q water (Milli-Q apparatus; Millipore Corp., Bedford, MA, USA). The mature coconuts (product of Tonga) were bought from local grocery store. All experiments were repeated a minimum of two times unless otherwise specified.

3.2 Methods

3.2.1 Preparation of coconut oil body membrane (COBM) material extracts and coconut skimmed milk material extracts (CSME)

The coconuts were cracked and deshelled manually to remove the coconut water. The fresh coconut kernel with adhering test was washed using potable soft water (20 °C) and cut into small pieces manually by a kitchen knife. Following this, the coconut kernel pieces were passed through an electric mincer to reduce the size. Potable soft water (20 °C) was added to the minced kernel at a kernel-to-water ratio of 4:1 (w/w). The mixture was then pressed in a pneumatic press (3.5 kg/cm², 10 min) in a double-layered cheese cloth bag to extract coconut milk. For separation of coconut cream the extracted coconut milk was passed through a cream separator (Model LWA 205, Westfalia Separator AG, Germany) working at 12,000 rpm resulting in a stream of coconut cream and skimmed milk. Both raw coconut cream and coconut skimmed milk were collected for further processing.

Coconut oil body membrane (COBM) material extracts

The raw cream was washed and diluted to final fat content 5% (w/w) with potable soft water (40°C) and passed through the cream separator to obtain final coconut cream. The washing process was repeated twice to remove skimmed proteins and sugars. The final cream was frozen to -18°C overnight and thawed to 40°C with stirring to break the oil bodies and release the fat. The thawed cream with disrupted oil body emulsion was centrifuged at 15000 g for 30 min, which resulted in three layers. The top layer consisted of free oil and was discarded. The bottom layer was transparent and likely to be devoid of any intact oil bodies or free oil and contained interfacial membrane materials of disrupted oil bodies. This layer was collected and the serum phases from different runs

were pooled and freeze dried to get coconut oil body membrane extracts (COBM). The middle layer consisted of unbroken oil body was collected and pooled with those from other runs and subjected to freeze-thaw treatment and centrifugation extraction again. When collected the bottom layer, a small amount of the middle layer was also removed to avoid any mixing among different layers. Fat, phosphorus, protein and moisture content of COBM were analyzed by the protocols described in following sections.

Coconut skimmed milk extracts (CSME)

Coconut skimmed milk obtained from coconut milk was collected and centrifuged at 15000 g for 10 min to sediment any insoluble materials. The transparent serum phase was collected and grey sediment was discarded. The centrifuged skimmed milk was dialyzed in distilled water by using dialysis membrane tubing (10 kDa MWCO) for 12 hours minimum at 4°C. This allowed removal of sugars in the coconut milk. The dialysed skimmed milk fraction were freeze-dried to obtain coconut skimmed milk extracts (CSME). Fat, phosphorus, protein and moisture content of CSME were analyzed by the methods, which were described in the following sections.

3.2.2 Preparation of emulsions stabilized by coconut oil body membrane (COBM) and coconut skimmed milk extracts (CSME)

Coconut skimmed milk extracts (CSME) formed emulsions and coconut oil body membrane (COBM) formed emulsions were prepared at four final protein concentrations (0.2, 0.4, 0.6 and 0.8%, w/w) and 20% oil concentration (Table 3.1).

Table 3.1 Composition of CSME and COBM emulsions

| Emulsions | Protein concentration in final emulsion (% , W/W) | Oil concentration in final emulsion (% , W/W) |
|------------------|--|--|
| CSME emulsions | 0.2 | 20 |
| | 0.4 | 20 |
| | 0.6 | 20 |
| | 0.8 | 20 |
| COBM emulsions | 0.2 | 20 |
| | 0.4 | 20 |
| | 0.6 | 20 |
| | 0.8 | 20 |

The COBM and CSME were dissolved in Milli-Q water and stirred at 40°C, for 2 hours to allow dissolution. The protein solutions were centrifuged at 10000 g for 10 min. For CSME, the top transparent water phase was collected for use for further experiments and sediment, which contained insoluble proteins was discarded. For COBM, solutions separated into three layers upon centrifugation (10000 g for 10 min). The top fat layer consisting of fat/unbroken oil bodies was carefully removed and discarded. The middle serum phase was collected as COBM solution for further emulsion formation and the bottom sediment pellet was discarded. The concentration of protein in the respective solutions was estimated using Bradford assay (Section 3.2.5.2). The COBM and CSME solutions were diluted to different concentrations to get final protein concentrations shown in Table 3.1.

The primary CSME and COBM emulsions were prepared by mixing the COBM and CSME solutions with 20% soybean oil and using a high shear mixer (Ultra-Turrax T18, IKA Works Inc., Wilmington, NC, USA) for 2 min. The primary emulsions were then passed two times through a microfluidizer (M-110EH, Microfluidics, Westwood, MA, USA) at 1200 PSI. The final emulsions were stored at 20°C up to 35 days after adding sodium azide (0.02%, w/w) as an antimicrobial agent.

3.2.3 Analysis of fat content

The fat content in sample was estimated by organic solvents extraction method described by Bligh and Dyer (1959). Briefly, 10 mL sample was added into 30 mL chloroform and methanol mixture (1:2, v/v) and blended for 2 min by using vortex mixer. 10 mL chloroform was then added and blending for 30 seconds, following which 10 mL Milli-Q water was added to the mixture and with vortexing for a further 30 seconds. The mixture was then transferred to a centrifuge tube and centrifuged at 3000 g for 10 min. After centrifugation, the mixture was separated into methanol layer and a heavier chloroform layer containing extracted neutral lipids. The bottom chloroform layer was carefully collected into a pre-weighed beaker and was left in a fume hood overnight to evaporate the chloroform. The amount of extracted fat left in beaker was weighed and expressed as percentage (w/v) of the sample.

3.2.4 Total phosphorus analysis

The total phosphorus in sample was determined by the Bartlett phosphate determination method, which was described by Dittmer and Wells (1969). This method is based on 2-step principle. Firstly, lipid sample is digested by perchloric acid to transfer organic phosphorus to inorganic phosphorus. In the second step, the inorganic phosphate is estimated by reaction with ammonium molybdate. The formation of phosphomolybdic acid is determined by using spectrophotometer (Genesys 10-S; Thermo Fisher Scientific Inc., USA). All test tubes and beakers used in the experiments were rinsed 10 times with Milli-Q water to avoid any contamination from phosphorous present in water.

A. Lipid sample digestion

Lipid sample and 0.4 mL 70% perchloric acid (w/v) were placed in test tube. The mixture was digested in an electric heating block at 180°C. During the digestion, about half of the tube extended outside of the heating block to allow reflux and condensation of acid back to the mixture. The digestion was stopped until the sample became colourless and cooled slowly to room temperature.

B. Determination of inorganic phosphate

2.4 mL ammonium molybdate solution and 2.4 mL Fiske and SubbaRow reducing solution were added into digested sample (contains up to 0.15 micromole phosphorus). A standard curve was prepared by diluting the 10 mM KH_2PO_4 stock solution to different concentrations as Table 3.2 and using these solutions in the assay instead of the lipid sample. All digested samples, standards and blank were heated in boiling water for 10 min and cooled to room temperature. The samples and standards were transferred into 2 mL quartz cuvettes (1 cm path length) and determined absorbance by using spectrophotometer (Genesys 10-S; Thermo Fisher Scientific Inc., USA) at 830 nm. Standard curve was shown in Figure 3.1.

Table 3.2 Concentrations of 10 mM KH_2PO_4 used for the preparation of the standard curve

| 10mM KH_2PO_4 (mL) | H_2O (mL) | Phosphorus in 0.1mL Solution (micromole) |
|--|---|---|
| 0 | 10 | 0 |
| 0.25 | 9.75 | 0.025 |
| 0.5 | 9.5 | 0.05 |
| 1 | 9 | 0.1 |
| 1.5 | 8.5 | 0.15 |

Reagent preparation

(1) 10 mM KH_2PO_4 stock solution: 1.361 g of was dissolved in 1 L Milli-Q water. Few drops of chloroform were added and stored at 4°C cool room.

(2) Ammonium molybdate solution: 4.4 g of ammonium molybdate was dissolved in 200 mL Milli-Q water. 14 mL concentrated sulfuric acid was added and made the final volume to 1 L with Milli-Q water.

(3) Fiske and SubbaRow reducing solution: Dissolve 1.217 g of Fiske and SubbaRow reducing reagent in 100 mL Milli-Q water. This reagent could be stored at cool room for 6-8 weeks.

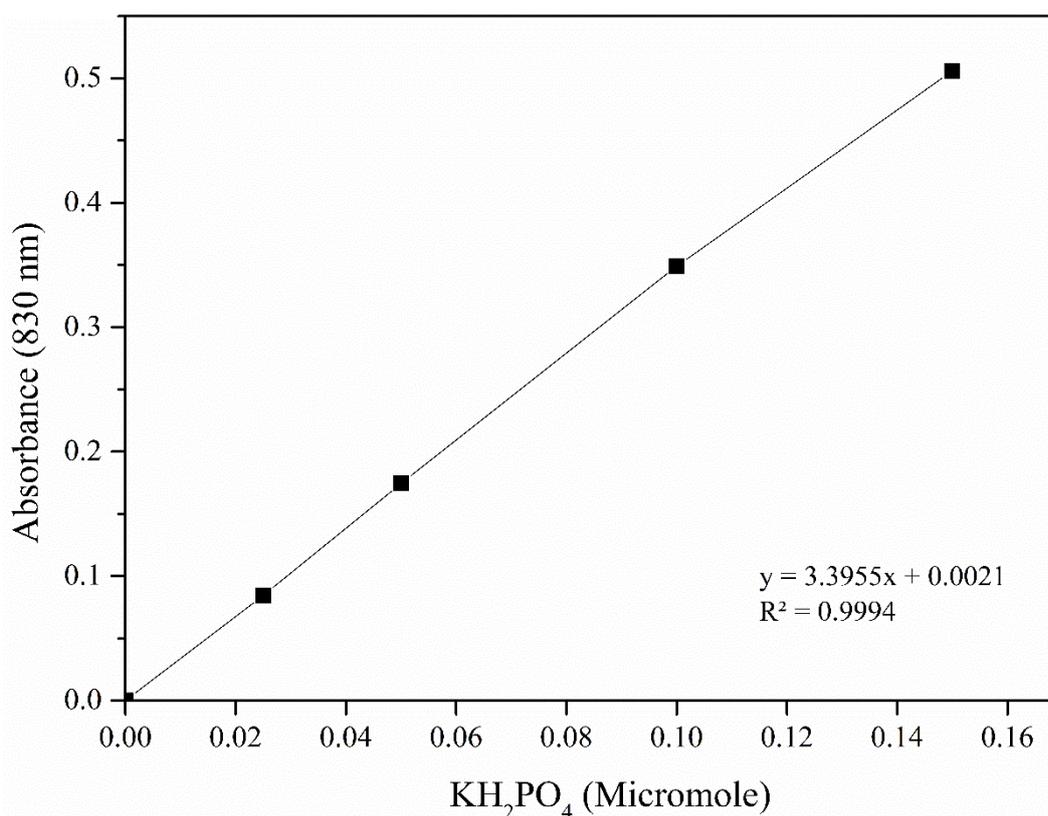


Figure 3.1 Standard curve showing absorbance at 830 nm for solutions showing different concentrations of potassium-dihydrogen orthophosphate when measured as per method described above.

3.2.5 Protein analysis

3.2.5.1 Protein content by the Kjeldahl method

The protein content in sample was determined by measuring the total nitrogen content in sample using Kjeldahl method (AOAC Official Method 991.20). The sample (approximately 0.125 g protein) was placed into Kjeldahl digestion tube, mixed with 25 mL concentrated H₂SO₄ and 2 Kjeltabs tablets (catalytic agent) (3.5 g K₂SO₄/3.5 mg Se). The Kjeldahl digestion tube was placed on a digestion block (Tecator digestion block DS20, Tecator, Sweden) maintained at 420°C until sample became colourless. 75 mL reverse osmosis treated (RO) water was added into the digestion tube after cooling. The digestion tube was set in a distillation unit (Kjeldahl system 1026, Tecator, Sweden) after adding concentrated alkali (40%, w/v NaOH). The alkali converted the (NH₄)₂SO₄ into free ammonia (NH₃). During distillation, the free ammonia escaped with steam into a flask, which contained 50 mL of 4% (w/v) boric acid with a few drops of phenolphthalein (colour indicator). The ammonia reacted with boric acid resulting in the formation of

ammonium borate complex which changed the colour of the solution from reddish to greenish blue. The ammonia in the complex was back titrated by using 0.1 M HCl until the colour of solution turned back to reddish or grey-mauve. The volume of used HCL was recorded and was used to calculate protein concentration by using following equations. Blank sample without any sample was also treated in the same way as above.

$$\text{Nitrogen \%} = \frac{(A-B) \times 1.4 \times 100}{C \times 1000} \quad (\text{Eq. 3.1})$$

$$\text{Protein (\%)} = \% \text{ Nitrogen} \times 6.25 \quad (\text{Eq. 3.2})$$

Where A = HCl used (ml), B = exact molarity HCL, C = weight of original sample (g)

This method of protein estimation has been used in this study to determine the total protein concentration in COBM and CSME.

3.2.5.2 Protein content by Bradford method

Protein concentrations in solutions were measured using Bradford method (Noble & Bailey, 2009). Bovine serum albumin (BSA) solution was selected as standard and prepared at different concentrations (0, 0.25, 0.5, 0.75, 1, 1.5 mg/mL). 50 μ L of standard solution or samples (protein concentration 0-1.5 mg/mL) were added into 2 mL Bradford reagent and incubated for 10 min at room temperature. The standards and sample were transferred into 2 mL disposable cuvettes (path length - 1 cm path) and determined absorbance by using spectrophotometer (Genesys 10-S; Thermo Fisher Scientific Inc., USA) at 595 nm. The standard curve showing the absorbance of different concentrations of BSA is shown in Figure 3.2 below.

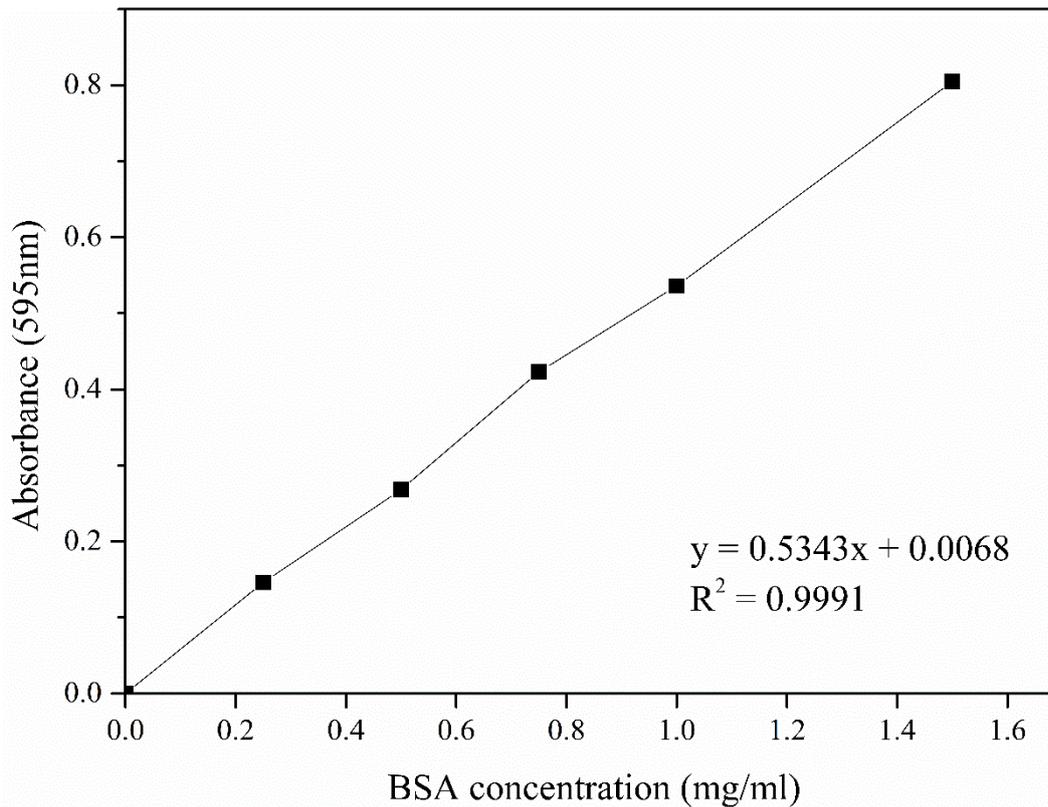


Figure 3.2 Standard curve for determination of protein concentration by Bradford assay

3.2.5.3 Protein solubility

To prepare 2.5% (w/w) CSME and COBM solutions, CSME and COBM were dispersed in Milli-Q water with stirring at 40°C over 2 hours. The samples were then centrifuged at 10000 g for 10 min. Protein concentrations in samples before centrifugation (total protein) and the supernatant obtained upon centrifugation (soluble protein) was measured using Kjeldahl method described in Section 3.2.5.1. The protein solubility (PS) was calculated by following equation:

$$PS\% = \frac{P_S \times 100}{P_T} \% \quad (\text{Eq. 3.3})$$

Where P_S = soluble protein content (g), P_T = total protein content (g).

3.2.5.4 Particle size distribution in extracts

Particle size distribution in protein solutions from CSME and COBM was measured by using Malvern Zetasizer Nano ZS (Model ZEN 3600, Malvern, Worcestershire, UK). The samples were placed into 2 ml quartz cuvettes (1 cm path length).

3.2.5.5 Solution optical density

The optical density of CSME and COBM solutions at different pH was measured by spectrophotometer (Genesys 10-S; Thermo Fisher Scientific Inc., USA) at 600 nm using 2 ml quartz cuvettes (1 cm path length). Milli-Q water was used as blank. All sample was measured without dilution.

3.2.5.6 Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis (SDS-PAGE) was used to investigate the molecular weight and type of proteins in the samples (Laemmli, 1970). The samples were analyzed under reducing or non-reducing conditions. The original emulsion (total protein in the emulsion), emulsion serum (protein present in emulsion serum phase) and emulsion oil body surface protein extracts (protein present on the droplet surface) were analyzed in this study.

(A) Sample preparation for SDS-PAGE

The proteins associated with oil bodies or emulsions were extracted using the method described by Tzen and Huang (1992). The emulsion samples were centrifuged at 13600 *g* for 20 minutes to obtain droplet/oil body rich phase and serum. 1 mL cream was mixed with 1 mL diethyl ether in 2 ml Eppendorf tube and centrifuged at 13600 *g* for 4 min. The upper ether layer was discarded and the residual ether layer was allowed to be evaporated. 750 μ L of chloroform and methanol mixture (2: 1, v/v) was added into aqueous followed by mixing (30 s) and centrifugation at 13600 *g* for 4 min. The interfacial layer was collected and mixed with 250 μ L Milli-Q water and again with 750 μ L chloroform-methanol mixture (2: 1, v/v). Finally, the sample was centrifuged at 13600 *g* for 4 min and the interfacial layer, which contained proteins associated with oil body emulsions was collected and resuspended in 0.5 mL Milli-Q water. For SDS-PAGE analysis, oil body surface protein or serum was mixed with SDS-PAGE reducing sample buffer and make the final protein concentration at approximately 1 mg/mL. The composition of PAGE buffer is shown in Table 3.3 below. For analysis under reducing conditions, the samples in reducing buffer was heated at 56°C for 15 min to allow reduction of disulfide bridges.

Table 3.3 Composition of SDS-PAGE sample buffer

| Buffer component | Composition (mL) | |
|--------------------------------|------------------|---------------------|
| | Reducing buffer | Non-reducing buffer |
| 25 % glycerol (w/v) | 6.25 | 6.25 |
| 0.5 M Tris-HCl buffer (pH 6.8) | 3.125 | 3.125 |
| 10 % SDS (w/v) | 5 | 5 |
| 0.1% Bromophenol blue (w/v) | 2.5 | 2.5 |
| Water | 6.875 | 6.875 |
| 1 M DDT | 1.25 | 0 |

(B) Preparation of gels and running conditions

The SDS-PAGE gel were prepared in-house using a Mini-Protean Tetra Cell (Bio-Rad Laboratories, Richmond, CA, USA) as per method described by Manderson, Hardman, and Creamer (1998). The composition of resolving and stacking gels is described in Table 3.4.

Table 3.4 Composition of SDS-PAGE resolving and stacking gel solutions

| Reagents | Resolving gel solution | Stacking gel solution |
|----------------------------------|------------------------|-----------------------|
| Milli-Q water | 2.02 mL | 3.05 mL |
| 1.5 M Tris-HCl buffer, pH 8.8 | 2.5 mL | 0 mL |
| 0.5 M Tris-HCl buffer, pH 6.8 | 0 mL | 1.25 mL |
| 10% SDS | 100 μ L | 50 μ L |
| Acrylamide (30%, w/v) | 5.3 mL | 0.65 μ L |
| Add before casting gel | | |
| TEMED | 5 μ L | 5 μ L |
| 10% APS (w/v) (freshly prepared) | 50 μ L | 25 μ L |

The gels were run at a constant voltage of 200 mV for 90 minutes until the dye reached the bottom of the plate. The run gels were stained by Coomassie brilliant blue solution (0.3% w/v, in 10 % (v/v) glacial acetic acid and 20 % (v/v) isopropanol for 1 hour and

destained by placing them in a solution containing 10% (v/v) iso-propanol and 10% (v/v) glacial acetic acid. Flat-bed scanner (Scanmaker i900, Microtek, Carson, CA, USA) was used to scan the gel.

3.2.6 Interfacial tension

The interfacial tension of CSME and COBM solutions was measured by tensiometer (KSV Instruments Ltd., Helsinki, Finland) as per method described by Ye, Zhu, and Singh (2013). The interfacial tension of solutions was determined by the pendant drop method with the time evolution. A droplet of solution (about 20 μL) was produced and hold by syringe needle tip (diameter 1.473 mm). Tensiometer continuously records the droplet volume, shape and area for 3 hours. The interfacial tension (γ) could be calculated by using following equation:

$$\gamma = \frac{\Delta\rho \times g \times R_0}{\beta} \quad (\text{Eq. 3.4})$$

Where $\Delta\rho$ refers to the density difference between two phases (density of water and soybean oil are 1 and 0.917 g/mL, respectively), g means gravitational constant, R_0 refers the radius of drop curvature at the apex, β means the shape factor, which could be calculated by Young–Laplace equation.

3.2.7 Emulsion characterization

CSME and COBM emulsions were prepared by the method described in Section 3.2.2. the measurements were done on emulsions without any treatment or either by adjusting the pH of emulsions 2 to 8 (using 0.1 M HCl, 1 M HCl and 0.1 M NaOH) or different concentrations of NaCl (0-500 mM).

3.2.7.1 Particle size distribution of emulsions

The droplet size distribution of emulsion was measured by static light scattering on a MasterSizer 2000 (Hydro MU, Malvern, Worcestershire, UK). The particle size measurements were carried out in two different conditions: The emulsion samples were dispersed in water, and then in sodium dodecyl sulphate (SDS) solution (1%, w/v in the final diluted emulsion solution). SDS could act as small molecular weight emulsifier to displace the protein adsorbing on the surface of droplets and disrupt the protein-protein interaction among droplets. The particle size, determined in water, indicated the “effective” particle size, which also includes the droplet flocs. However, the particle size

measured with SDS is regarded as the “primary” particle size (Tangsuphoom & Coupland, 2009). The difference in the particle size distribution in water and SDS solutions indicates flocculation or coalescence of droplets in the emulsions.

Sauter-average diameter $d_{(3,2)}$ and volume-mean diameter $d_{(4,3)}$ were determined at laser obscuration between 10 and 15%. The refractive index of soybean oil and water used in the protocols for measurement were 1.456 and 1.33, respectively. The average of triplicate measurements was calculated as mean particle size. The equation of $d_{(3,2)}$ and $d_{(4,3)}$ are followed:

$$d_{(3,2)} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (\text{Eq. 3.5})$$

$$d_{(4,3)} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (\text{Eq.3.6})$$

where n_i is the number of particles and d_i is the diameter of particles.

3.2.7.2 ζ - potential

Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) equipped with a 4 mW He/Ne laser with an output wavelength of 633 nm, was used to measure the ζ -potential of emulsions. To prevent multiple light scattering from droplets, the emulsion samples were diluted to a final droplet concentration of 0.01% (w/w) with buffer solution at the different pH and/or NaCl concentrations. The samples were placed in the electrophoresis cell (DTS 1060C, Malvern Instruments Ltd., Malvern, UK) and the refractive index of soybean oil and water are 1.456 and 1.33, respectively. The ζ - potential was measured at least 12 times for each sample at 25°C. The ζ - potential values were calculated by the Smoluchowsky mathematical model. Average values were calculated from triplicate measurements.

3.2.7.3 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (Model Leica SP5 DM6000B, supplied by Leica Microsystems, Heidelberg, Germany) was used to investigate the microstructure of emulsions using the staining protocols described by Gallier, Gordon, and Singh (2012). Nile Red (1 mg/mL in acetone), Fast Green FCF (1 mg/mL in Milli-Q water) were used to selectively stain neutral lipids and proteins respectively. The distribution of

phospholipids on the surface of oil droplets was investigated by staining using the dyes Lissamine™ rhodamine B (Rd-DHPE, 1 mg/mL in chloroform). The emulsion samples were diluted to a final oil concentration of 5% (w/v) with buffer solution at the same pH and NaCl content of the sample. 100 µL diluted sample was mixed with Nile Red (2:100, v/v), Fast Green FCF (6:100, v/v), Lissamine™ rhodamine B (Rd-DHPE, 5:100 v/v). The stained emulsion sample was placed on a concave microscope slide and covered by cover slip (0.17 mm thick), avoiding an air bubble between sample and slip. The freshly prepared sample slide was immediately examined by confocal laser scanning microscope with 63-mm oil immersion objective lens.

3.2.7.4 Cream stability measurement

7 g of emulsion was placed into a transparent screw-top glass tubes and sealed tightly. The tubes were allowed to be stored at room temperature without disturbance. Over time the emulsion separated into top opaque layer (containing the droplets) and bottom transparent/slightly turbid layer (serum phase). The height of the emulsions (H_T) at different times (0 h to 36 h). The creaming index was calculated as following equation (White et al., 2008):

$$\text{Creaming Index} = 100 \times \frac{H_T}{H_E} \quad (\text{Eq. 3.7})$$

All samples used for cream stability measurement was freshly prepared and at least duplication was carried out.

3.2.7.5 Protein content on oil droplet surface

Emulsion serum was separated from original emulsion by centrifugation at 13600 g for 20 min. P_T and P_S were measured by Kjeldahl method described in Section 3.2.5.1 and Bradford method described in Section 3.2.5.2, respectively. Protein content on oil droplet surface was calculated as follow:

$$\text{Ratio of surface protein in total protein (\%, w/w)} = \frac{100 \times (P_T - P_S)}{P_T} \quad (\text{Eq. 3.8})$$

Where P_S refers to protein content in emulsion serum, P_T refers to protein content in total emulsion.

3.2.8 Enzymatic studies to investigate the surface composition of emulsions

The enzymatic studies were carried out to determine the surface structure of the emulsions from extracts and to predict their behaviour during digestion conditions. The *in vitro*

digestion protocol described by Minekus et al. (2014) was used in this study. It is worth noting that it is not a full-scale digestion. Instead of that, the surface of emulsion droplets was perturbed by using enzymes pepsin, trypsin and phospholipase A₂, separately. The composition of concentrated simulated gastric fluid (SGF) and concentrated simulated intestinal fluid (SIF) was listed in Table 3.5.

Table 3.5 Compositions simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) for enzyme studies

| Composition | Stock conc. (mol/L) | SGF (mL) ^a | SIF (mL) ^a |
|---|---------------------|-----------------------|-----------------------|
| KCl | 0.5 | 6.9 | 6.8 |
| KH ₂ PO ₄ | 0.5 | 0.9 | 0.8 |
| NaHCO ₃ | 1 | 12.5 | 42.5 |
| NaCl | 2 | 11.8 | 9.6 |
| MgCl ₂ (H ₂ O) ₆ | 0.15 | 0.4 | 1.1 |
| (NH ₄) ₂ CO ₃ | 0.5 | 0.5 | / |
| For pH adjustment | | | |
| HCl | 6 | 1.3 | 0.7 |

^aThe pH of SGF was pH 2 and that of SIF was pH 7

Pepsin treatment

2 mL emulsion sample was mixed with 6.4 mL 1.25× SGF and 2.5 μL 3M CaCl₂ (reach 0.075 mM in final digestion sample). The pH of the mixture was adjusted to pH 2 by 0.1 M HCl and to the final volume was made to 9 mL with water. The mixture was incubated in 37 °C water bath with vibration for 15 min. After incubation, 950 μL pepsin solution (2000 U/mL in the final mixture) was added into the mixture. 10 mL final digestion sample was incubated in 37 °C water bath with shaking for 1 h. Particle size was measured immediately upon completion of the incubation by using MasterSizer (Hydro MU, Malvern, Worcestershire, UK). The rest of the mixture was adjusted to pH 7 by 0.1 M NaOH, in order to inhibit the pepsin. The microstructure of emulsions was determined by using confocal laser scanning microscopy (Model Leica SP5 DM6000B, supplied by Leica Microsystems, Heidelberg, Germany).

Trypsin treatment

2 mL emulsion sample was mixed with 6.4 mL 1.25× SIF and 10 µL 0.3M CaCl₂ (reach 0.03 mM in final digestion sample). The pH of the mixture was adjusted to pH 7 by 0.1 M NaOH and made mixture volume to 9.7 mL with water. The mixture was incubated in 37 °C water bath with vibration for 15 min. After incubation, 275 µL trypsin solution (100 U/mL in the final mixture) was added into the mixture. 10 mL final digestion sample was incubated in 37 °C water bath with vibration for 1 h. 300 µL trypsin inhibitor (2 mg/mL) was added to the digested sample to inhibit the trypsin. The particle size and microstructure of emulsions was determined by using MasterSizer 2000 and confocal laser scanning microscopy.

Phospholipase treatment

2 mL emulsion sample was mixed with 6.4 mL 1.25× SIF and 33 µL 3M CaCl₂. The pH of the mixture was adjusted to pH 8.5 by 0.1 M NaOH and made mixture volume to 9mL with water. The mixture was incubated in 37 °C water bath with vibration for 15 min. After incubation, 950 µL phospholipase A₂ (≥ 20 units/mg) solution (2 mg/mL) was added into mixture. 10 mL final digestion sample was incubated in 37 °C water bath with vibration for 1 h. The particle size and microstructure of emulsions was determined by using MasterSizer 2000 and confocal laser scanning microscopy.

3.2.9 Statistical analysis

The obtained data were statistically analyzed using unpaired Student's t-test (two-tailed). The significant differences between two mean values were considered at $p < 0.05$.

4. Characterization of coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM)

4.1 Introduction

Coconut oil body membrane materials present themselves as ideal materials for emulsification due to their ability to stabilize oil bodies under various conditions. They have potential applications in food and pharmaceutical formulations for encapsulation of bioactive compounds. Coconut proteins and phospholipids naturally stabilize the coconut oil body, which indicates that coconut oil body membrane may act as a good food emulsifier. Meanwhile, most of the the coconut proteins remained in the coconut skimmed milk, which could be a good source as emulsifiers. The interfacial properties of membrane materials from coconut oil body have been re-visited and investigated in detail (Dave et al., 2019). In this chapter, the differences of the proximate compositions and properties of coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM) from coconuts were investigated.

4.2 Results

4.2.1 Composition of coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM)

The proximate composition of the freeze-dried coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM) are presented in Table 4.1. The moisture, protein and total phosphorus contents in CSME were higher than that in COBM, while the fat content in COBM was remarkably higher than that in CSME, probably resulting from the residual fat content from the material preparation steps.

Table 4.1 Composition of coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM)

| Items | CSME | COBM |
|---------------------------------|-------------|--------------------------|
| Moisture (% , w/w) ^a | 15.0 ± 0.01 | 6.4 ± 0.01 [*] |
| Protein (% , w/w) ^a | 41.4 ± 0.03 | 16.1 ± 0.03 [*] |
| Fat (% , w/w) ^a | <0.1 | 36.1 ± 0.02 [*] |
| Phosphorus (mg/g) ^a | 15.3 ± 0.96 | 3.5 ± 0.45 [*] |
| Others (% , w/w) ^b | 43.6 | 41.4 [*] |

^a Values are average from triplicate determinations. ^b Estimated by calculation. * represents significant differences (p < 0.05) between samples.

4.2.2 Protein solubility of CSME and COBM

The soluble protein content in the centrifuged CSME and COBM solutions was measured by dissolving the extracts in water followed by the estimation of protein content before and after centrifugation. The protein solubility of CSME and COBM was shown in Table 4.2. As can be seen, the COBM had higher protein solubility than CSME. The most of proteins from COBM remained soluble in water.

Table 4.2 Protein content in extracts and their solubility after centrifugation

| Items | Total protein in extracts (% , w/w) | Protein in centrifuged solution (supernatant) (% , w/w)* | Protein solubility (%) |
|-------|-------------------------------------|--|------------------------|
| CSME | 41.2 | 30.0 | 72.8 |
| COBM | 16.5 | 15.6 | 94.5 |

* Samples centrifuged at 10000 g for 10 min at 20°C

4.2.3 Characterization of proteins in CSME and COBM

To identify the soluble proteins present in CSME and COBM, the samples of CSME and COBM solutions after centrifugation were analysed by reducing SDS-PAGE. In comparison, the freshly extracted coconut oil body proteins (Dave et al., 2019) were also presented in Figure 4.1.

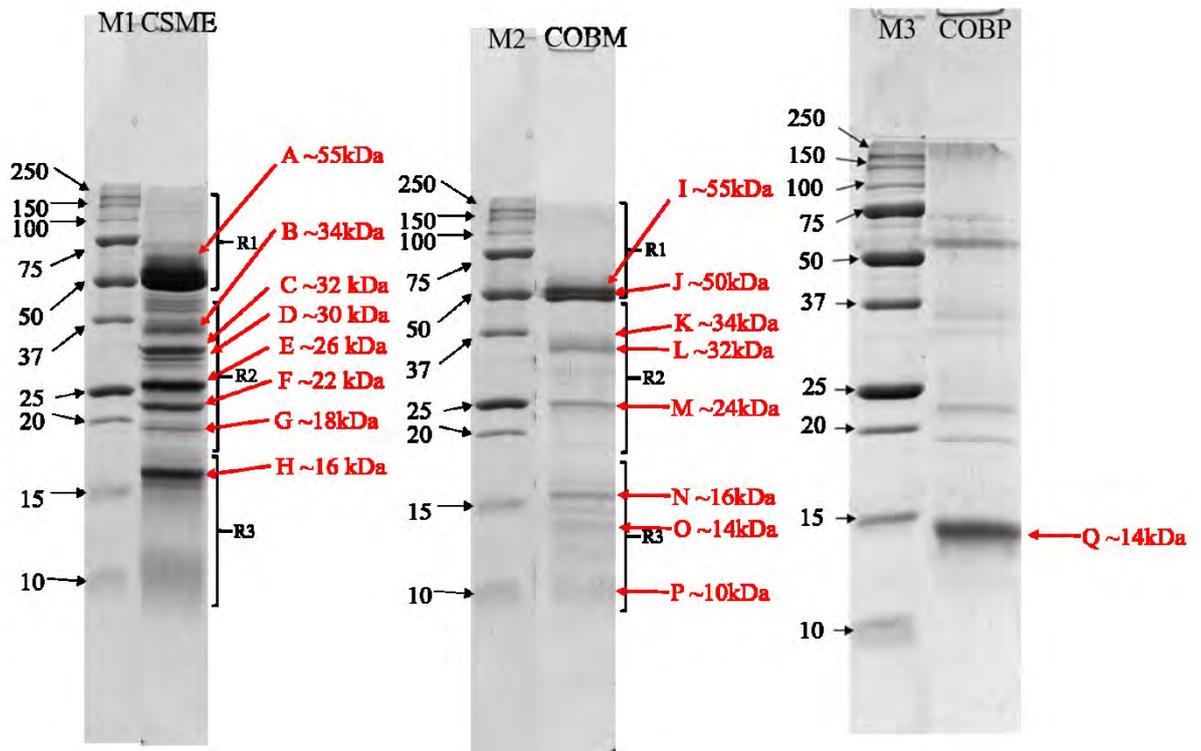


Figure 4.1 Protein composition of coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM). M_1 , M_2 and M_3 are molecular mass markers. CSME was coconut skimmed milk extracts solution and COBM is coconut oil body membrane solution. COBP refers to freshly extracted coconut oil bodies, adapted from Dave et al. (2019). For the description of bands marked A to Q, see text.

CSME solution showed at least 13 polypeptide bands between 10 and 250 kDa, which were: 1 band at approximately 55 kDa (Region R1); 3 distinct bands between 37 kDa and 50 kDa (Region R2); 5 bands between 20 kDa and 37 kDa (Region R2) and 1 band at around 20 kDa (Region R3), where the most prominent 8 bands are marked (A-H). In comparison, the COBM solutions showed at least 8 distinct bands (I-P) between, while the most prominent bands were 2 bands at approximately 55 kDa (Region R1), 3 bands between 20 kDa and 50 kDa (Region R2); and around 3 bands in region R3 with molecular weights ≤ 15 kDa (Region R3). In natural coconut oil body extracted proteins (COBP), the major molecular weight was around 14 kDa (band Q).

Figure 4.1 shows that there were distinct differences in the protein present in the CSME and COBM extracts. In region R1, only 1 band (about 55 kDa) was observed in CSME

but the COBM showed two distinct bands. In region R2, at least two bands (between 37 and 25 kDa), presented in COBM and around 9 bands present in CSME were absent. The differences in the protein profiles in Figure 4.1 indicate that the protein compositions in CSME and COBM were different.

4.2.4 Effect of pH on CSME and COBM solutions

To characterize the coconut skimmed milk extracts (CSME) solution and coconut oil body membrane (COBM) solutions, these 2 types of solutions (2.5% w/w) were prepared and adjusted to different pH values (2 to 8) by using 1 M HCl and 0.1 M NaOH. The effect of pH on the changes in the physicochemical characteristics was then investigated by light scattering techniques.

The ζ - potential CSME and COBM solutions at different pH values are shown in Figure 4.2. The pH and ζ - potential of freshly prepared CSME and COBM solutions in water were pH 6.3 and -7.2 mV, and pH 8 and -29 mV, respectively. The isoelectric pH of CSME proteins (approximately pH 4.2) was lower than that of COBM (pH 5). At 2 pH terminal point (pH 2 and 8), the ζ - potential of for COBM solution (about +29 and -24.5 mV, respectively) were higher than that of CSME solution (about +7.5 and -13.6 mV, respectively). When $\text{pH} < \text{pI}$, the ζ - potential of both solutions became less positive with the increase in pH and reached 0 mV, at their isoelectric points. Beyond the isoelectric pH, the ζ - potential of 2 solutions increased (more negative) with an increase in pH. The changes in the ζ - potential (per unit of pH) were more for COBM solution as compared to the CSME solution.

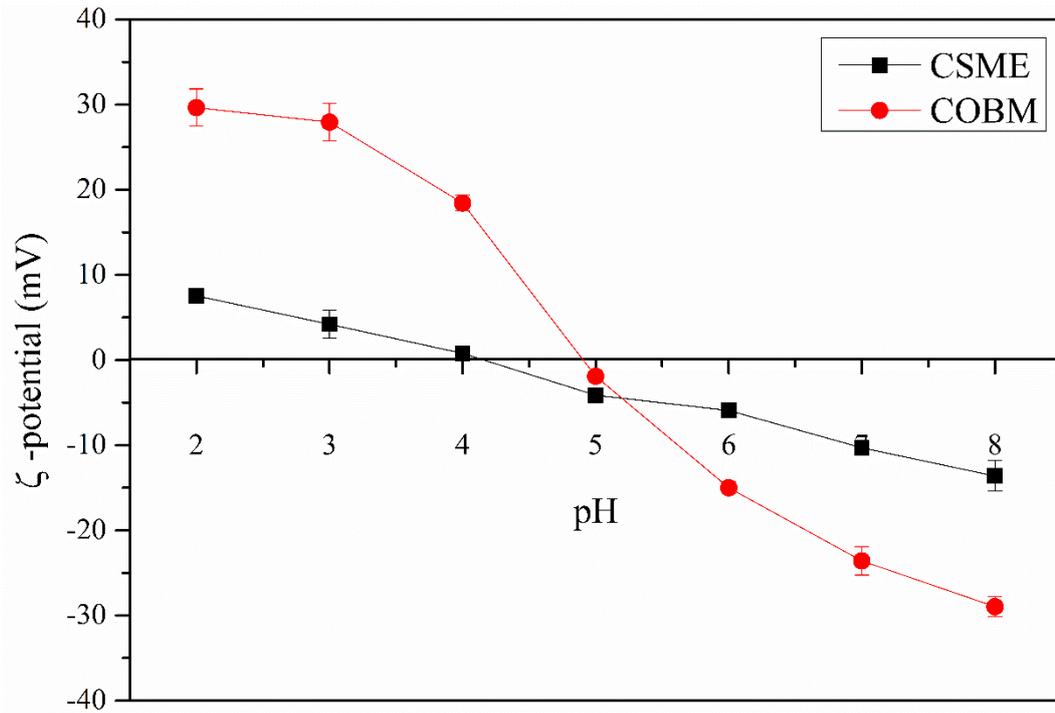


Figure 4.2 Effect of pH on the ζ - potential of coconut skimmed milk extracts (CSME) solution and coconut oil body membrane (COBM) solution

The particle size of CSME and COBM solutions at different pH (2-8) are shown in Figure 4.3. The particle size of proteins in CSME solution was approximately 1158 nm when dissolved in water without pH adjustment (pH 6). With a decrease of pH from 6 to 4, the particle size significantly increased and reached a maximum point (about 8317 nm) at pH 4 (near its pI). With the further lowering the pH, particle size decreased gradually. Above pH 6, the particle size increased only slightly.

The particle size of proteins in the COBM solution was about 300 nm without pH adjustment (pH 8). At the extremes of pH, from 2 to 4 and 6 to 8, the particle size of COBM remained unaffected (at approximately 300 nm), Between pH 4 and 6, the particle size increased, reaching a peak (at approximately 16400 nm) at its isoelectric point (pH 5) (Figure 4.2).

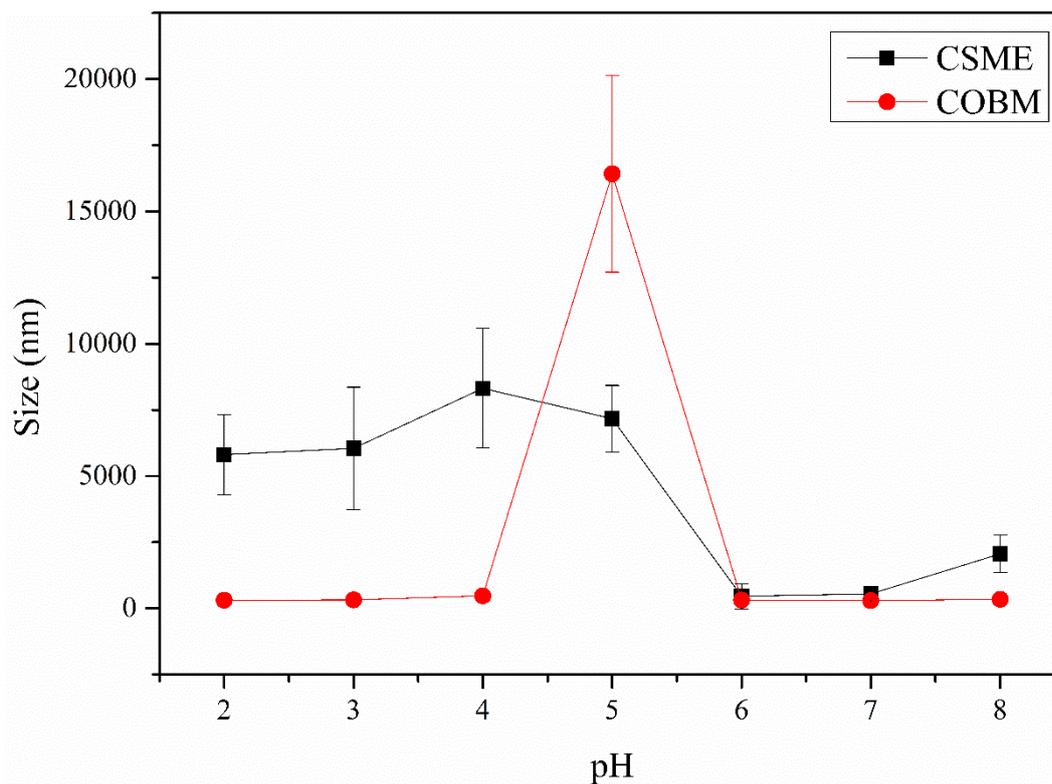


Figure 4.3 Effect of pH on the size of coconut skimmed milk extracts (CSME) solution and coconut oil body membrane (COBM) solution

The optical density (OD) of CSME and COBM solutions at different pH (2-8) were shown in Figure 4.4. The highest OD of both COBM and CSME solutions were at their pI (pH 4 to 5). The OD of CSME solutions remained relatively high at all pH values below the pI, but remained low at > pH 6, indicating the proteins were stable > pH 6. In comparison, at pH above or below the pI, the OD for COBM remained low.

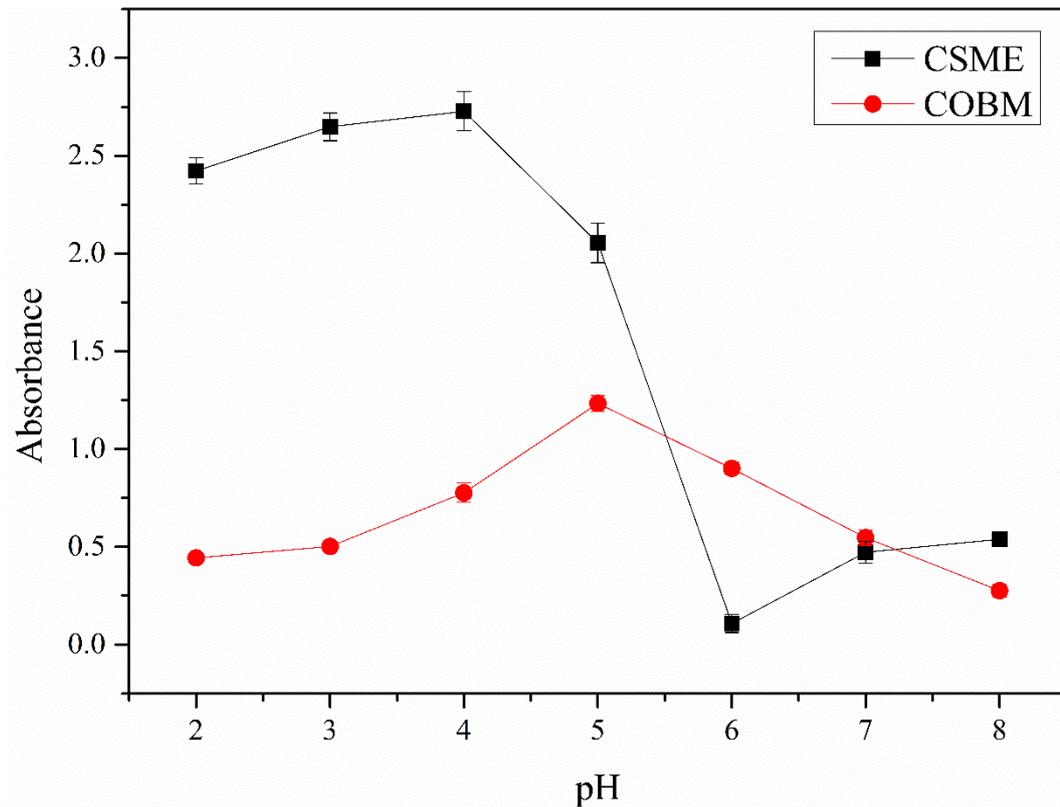


Figure 4.4 Effect of pH on the optical density of coconut skimmed milk extracts (CSME) solution and coconut oil body membrane (COBM) solution

4.2.5 Interfacial tension

The interfacial tension between soybean oil and Milli-Q water, CSME and COBM solution at 0.4% and 0.8% (w/w) protein concentrations were measured by pendant-drop tensiometry (Figure 4.5). In the pure water-oil system, Milli-Q water as drop phase, the interfacial tension was initially about 19.2 mN/m and quickly stable at about 17.2 mN/m, which is in agreement with previous reports (Fisher, Mitchell, & Parker, 1985). The interfacial tension of water was higher than that of CSME and COBM solutions. The interfacial tension of CSME solutions (0.4 and 0.8%, w/w, protein) decreased from about 12.4 and 10.4 to 5.8 and 5.4 mN/m, respectively. The initial and final interfacial tension of COBM (0.4 and 0.8%, w/w, protein) were lower than that of CSME, decreased from about 8.8 and 8.2 to 5.1 and 4.8 mN/m.

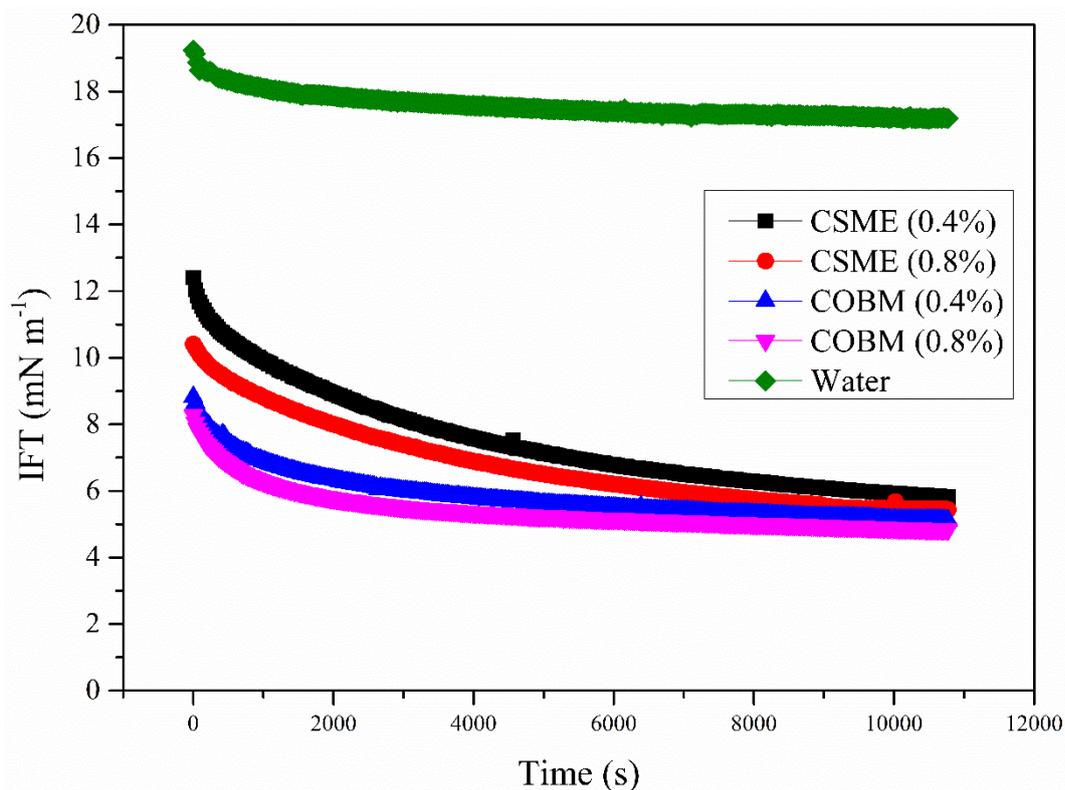


Figure 4.5 Interfacial tension of Milli-Q water, coconut skimmed milk extracts (CSME) solution and coconut oil body membrane (COBM) solution at 0.4% and 0.8% (w/w) protein concentrations

4.3 Discussion

The CSME showed comparatively higher proportion of proteins than COBM. This is expected since most of the coconut milk proteins are retained in the coconut skimmed milk upon centrifugation (Balasubramaniam & Sihotang, 1979). In comparison, the lower protein content in the COBM extracts indicates that these proteins may largely be associated with oil bodies in coconut milk.

The presence of total phosphorus (organic and inorganic) in the CSME and COBM may indicate the phospholipids are contained in the extracted materials. The total phosphorous content of CSME was higher than that of COBM, which may indicate a comparatively more phospholipids in CSME than COBM. The high phosphorous content in the CSME may be possibly caused by strong mechanical centrifugation and washing, which disrupted the oil bodies and resulted in a part of interfacial phospholipids was released in the aqueous phase (Walstra, 1985). Zheng, Jiménez-Flores, and Everett (2013) showed

that the washing and centrifugation had an effect on breaking milk fat globule membrane (MFGM). In their study, the ζ - potential of washed milk cream increased from -12.24 to -13.04 mv, which may be caused by that outer phospholipid layer was broken and inner electron-dense protein layer was exposed, which indicates the damage of MFGM broken during centrifugation. The phospholipids distribution has been reported for bovine milk with up to 52 % present in skimmed milk and around 45 % in the cream phase (McDowall, 1953). McDowall (1953) reports that the phospholipids content in butter oil and buttermilk prepared by freeze-thaw treatment and churning were 0.182 % and 0.187 % (w/w), respectively. This suggests that phospholipids may release to both water and oil phase and it could be a possible reason for low phosphorus content in COBM.

Another possible reason for the higher phosphorus content in CSME could be the high inorganic phosphorus present in coconut milk (about 1mg/g total phosphorus in coconut milk) or contaminating phosphorus in the water used during manufacturing.

The functional properties of proteins from coconut skimmed milk and coconut cream proteins have been previously investigated. Onsaard et al. (2005) extracted coconut skimmed protein milk isolate (CSPI) and coconut skimmed milk protein concentrate (CSPC) by isoelectric precipitation and ultrafiltration, respectively. The composition of CSPC was close to that of CSME. The main compositions of these materials are shown in Table 4.3. In comparison, Onsaard et al. (2006) extracted coconut cream protein (CCP) by the method similar to that used in our study for COBM (freeze-thawing). Although, these authors reported a higher yield of proteins and lower fat in the final CCP powder (Table 4.3), which may be explained by the use of hexane to rinse the freeze-thawed cream materials to remove residual oil. In this study, no hexane wash was used to avoid any solvent-extraction step.

The data in Table 4.1 also indicates that both CSME and COBM contained other components, such as fat and carbohydrates (from coconut kernel or the testa). The studies discussed above note high carbohydrate contents of approximately 36.6 % and 14.7 % (w/w) in the CSPC and CCP, respectively. The difference between the compositions of CSME and COBM may affect the emulsion properties formed by these materials. Nevertheless, proteins may remain the main component responsible for the emulsification.

Table 4.3 Proximate compositions of coconut skimmed protein milk isolate (CSPI), coconut skimmed milk protein concentrate (CSPC) and coconut cream protein (CCP) (% , w/w) (Onsaard et al., 2005, 2006)

| Constituents | CSPI | CSPC | CCP |
|---------------|-------------|-------------|--------------|
| Moisture | 2.69 ± 0.02 | 6.5 ± 0.30 | 3.37 ± 0.16 |
| Protein | 59.6 ± 0.60 | 45.6 ± 0.1 | 75.27 ± 0.97 |
| Fat | 28.5 ± 0.50 | 0.79 ± 0.08 | 2.45 ± 0.24 |
| Ash | 5.25 ± 0.02 | 8.84 ± 0.02 | 4.24 ± 0.10 |
| Carbohydrate* | 3.9 ± 1.00 | 36.6 ± 0.06 | 14.67 ± 0.67 |

* Estimated by the difference

From Figure 4.3 and 4.4, COBM and CSME reached the highest protein solubility at pH 8 and 6, respectively. The differences in solubility of proteins in the two extracts may result from the type of proteins or their interaction with other components such as minerals and polar lipids.

The SDS-PAGE analysis of coconut milk emulsion from other studies reported 7 major bands at approximate molecular weights of 55, 33, 25, 21, 20, 18 and 16 kDa (Balachandran & Arumugan, 1992; Benjakul et al., 2016; Garcia et al., 2005). The bands at these molecular weights were showed in the SDS-PAGE of CSME, indicating that CSME contained most of the proteins present in original coconut milk emulsion. Only 8 major bands presented in the SDS-PAGE of COBM, which either absent or lighter in intensity in the SDS-PAGE of COBM. This suggests that the proteins in CSME and COBM were different and not all the proteins in whole coconut milk were associated with oil bodies.

Previous studies have reported that the main proteins in coconut milk consist of globulins (11S and 7S) and albumins (Kwon et al., 1996; Tangsuphoom & Coupland, 2009). The most prominent bands of CSME was at 55 kDa, which may correspond to the major coconut protein (11S globulin) with 55 kDa molecular weight, a hexamer consisting of a 32 to 34 kDa acidic subunits and a 22 to 24 kDa basic subunits (Garcia et al., 2005; N.Angelia et al., 2010; Tangsuphoom & Coupland, 2008). It is possible that the bands at 16 and 22 kDa in CSME SDS-PAGE gel may refer to of 7S globulins (Garcia et al., 2005).

The coconut milk albumins have molecular weights of approximately 18, 26 and 55 kDa (Garcia et al., 2005; Kwon et al., 1996; Tangsuphoom & Coupland, 2009). Thus, it is possible that the protein bands at 18, 26 and 55 kDa bands in CSME PAGE gel were albumins.

Tangsuphoom and Coupland (2009) and Rasyid et al. (1992) reported that the major proteins associated with coconut cream droplet interface were globulins, which may suggest that the most of 55 kDa protein in COBM PAGE gel was likely to be globulins. The other 3 major bands (16, 24 and 34 kDa) in COBM PAGE gel may also refer to globulin (Garcia et al., 2005). Although it is possible that 55 kDa band had contaminant proteins (such as albumin) of coconut skimmed milk. Further studies are necessary to ascertain the nature of the exact type of proteins found in the CSME and COBM.

Generally, the molecular weight range of oil body protein (oleosin) in oilseeds is 15 to 26 kDa (Huang, 1996; Tzen, Cao, Laurent, Ratnayake, & Huang, 1993). Regalado et al. (2008) and Dave et al. (2019) reported that the coconut oleosin's molecular weight was 14.4 kDa and 11 kDa and the major oleosin was around 14 kDa. From the SDS PAGE gel, COBM proteins exhibited a few bands with a molecular weight between 10 to 15 kDa, which may indicate that the coconut oleosin proteins were most likely extracted with the COBM.

The differences in the proteins of CSME and COBM had a significant influence on their particle size, ζ - potential and optical density in aqueous solutions. At a given pH, the ζ - potential was higher in COBM than in CSME solutions. This may be explained based on the differences in the composition of charged amino acid groups and the structure of the proteins present in the solutions.

Both CSME and COBM showed high solubility at $\text{pH} > \text{pI}$. This could be explained a strong electrostatic repulsion at $\text{pH} > \text{pI}$, which prevented protein-protein interactions, in turn resulting in high protein solubility (Hall, 1996). In comparison, the neutralization of charges at pI facilitated protein-protein interactions (Pelegri & Gasparetto, 2005), which was indicated by the increase in the particle size and high optical density in samples. At $\text{pH} < \text{pI}$, the particle size of CSME was similar to that at $\text{pH} 7$, while the CSME had a high particle size and remained turbid at all $\text{pH} < \text{pI}$. It is possible that CSME solutions

at pI had a very strong aggregation of proteins and when at $\text{pH} < \text{pI}$ the relatively low ζ -potential of CSME solutions ($< +10 \text{ mV}$) may be not able to provide enough electrostatic force to separate their aggregation.

Both CSME and COBM reduced the interfacial tension of water. The lower interfacial tension values of COBM than that of CSME may indicate that COBM had better capacity to reduce the interfacial tension and had better performance in terms of emulsification (McClements, 2015; Thaiphanit & Anprung, 2016), since less free energy is required to form an emulsion at lower interfacial tension (Garti, 1999). Meanwhile, higher protein concentration induced lower interfacial tension, which may indicate that more CSME or COBM in emulsion formulation may facilitate the emulsion formation. The relatively lower ability of CSME extracts to lower the surface tension of water than COBM suggests that the higher total phosphorous content detected through the inorganic phosphorous assay may have resulted from contamination from non-phospholipid sources of phosphorous.

4.4 Conclusions

Overall, both CSME and COBM extracts consisted of proteins and phospholipids. The types of protein varied in the two extracts, as noted by the SDS-PAGE analysis. The difference of protein types and ζ -potential induced the different performance of particle size and optical density (aggregation), when changed pH conditions. The ζ -potential of COBM was stronger than that of CSME at all pH values. The difference in the surface tension properties of COBM and CSME solutions suggested that COBM was more surface active than CSME.

5. Characterization of the emulsions stabilized by coconut skimmed milk extract (CSME) or coconut oil body membrane (COBM)

5.1 Introduction

The compositions and the properties of coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM) was investigated in the previous chapter. In this chapter, the emulsions stabilized by CSME or COBM have been studied and their surface membrane structure has been characterized.

5.2 Results

5.2.1 Emulsion protein analysis

The protocols used in this chapter have been described in Chapter 3. CSME emulsions and COBM emulsions were prepared at protein concentrations, 0.2%, 0.4%, 0.6% and 0.8% (w/w) (Table 3.1), using the method described in Section 3.2.1 and Section 3.2.2. After emulsions were prepared, the protein content in serum phase was measured after separating the emulsion droplets out by centrifugation at 13600 g for 20 minutes at 20 °C. The droplet surface protein content was then calculated (Table 5.1). When the protein concentration of CSME and COBM emulsions increased from 0.4 to 0.8%, the fraction of surface proteins on CSME and COBM emulsion droplets increased from approximately 75 % and 79 % to 84.7% and 82.3%, respectively. This indicates that most of the proteins were associated with the emulsion droplets.

Table 5.1 Distribution of protein concentrations in coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM) emulsions

| Items | Protein content (%, w/v) | Protein in serum phase (%, w/v) | Ratio of surface protein in total protein (%, w/w) |
|----------------|-----------------------------|------------------------------------|---|
| CSME emulsions | 0.4 | 0.122 | 75.6 ^a |
| | 0.8 | 0.153 | 84.7 ^b |
| COBM emulisons | 0.4 | 0.105 | 79.1 ^c |
| | 0.8 | 0.177 | 82.3 ^d |

Different lower case letters represent significant differences ($p < 0.05$) between samples

The protein composition of CSME and COBM emulsions and their serum and cream phases obtained after centrifugation at 13600 g for 20 min at 20°C was analyzed by SDS-PAGE (Figure 5.1). The composition of proteins in the CSME and COBM emulsions (Lane 3, A and B), were similar to the proteins of CSME and COBM (lane 2, A and B).

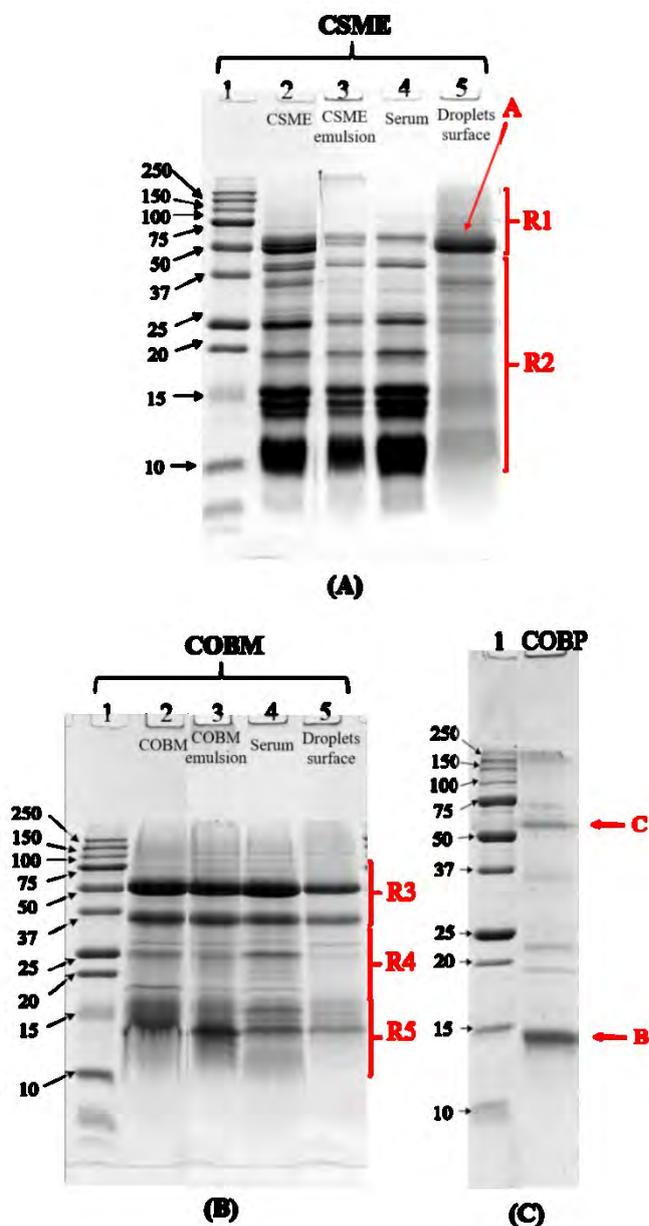


Figure 5.1 SDS-PAGE of interfacial proteins in (A): coconut skim milk extract (CSME); (B): coconut oil body membrane (COBM) emulsion droplets; and (C) freshly extracted coconut oil body protein (COBP). 1: Marker, 2: CSME or COBM solution (1mg/mL), 3: CSME or COBM emulsion, 4: Serum phase (after centrifugation at 13600 g for 20 min), 5: Interfacial proteins from emulsion droplets. Image in (C) adapted from Dave et al. (2019)

In the SDS-PAGE of CSME emulsion (Figure 5.1, A), most of the protein bands in the CSME emulsion (lane 3) were found in its serum phase (lane 4). The distinct differences could be noted in the protein composition of serum (lane 4) and the proteins associated with the oil droplets (lane 5). In Region R1, band A (around 50 kDa) was missing in serum protein (lane 4) and found in droplet surface protein (lane 5). In Region R2, the bands around 37, 25, 15 and 10 kDa, found in serum protein (lane 4) were missing in droplet surface protein (lane 5) or had worse intensity than serum protein. Although there were several bands were found in surface proteins (lane 5), but the 50 kDa band was the most prominent band.

In the SDS-PAGE of COBM emulsion (Figure 5.1, B), most of protein bands in the COBM emulsion (lane 3) presented in its serum phase (lane 4). In comparison, the most differences could be noted in the protein composition of serum (lane 4) and proteins associated with the oil droplets (lane 5). In Region R4 (between 20 to 35 kDa), most of the band presented in serum protein were missing in droplet surface protein. In Region R3 and R5, there were only 3 prominent bands (around 55, 37 and 15 kDa) in surface protein (lane 5), which were also presented in serum protein (lane 4). However, in Region R5 (below 20 kDa), the other bands in serum protein were missing in surface protein or had a stronger intensity than that in surface protein.

The coconut oil body protein (COBP) was extracted from natural coconut oil body in our lab (Dave et al., 2019), shown in Figure 5.1, C. The most dominative molecular weight (band B) was around 15 kDa. Meanwhile, the other band (band C) at approximately 55 kDa was observed, but at a much lower intensity than the one at 15 kDa. These two bands also appeared in the surface proteins of COBM emulsion (Figure 5.1, B, lane 5). These results may suggest that the natural coconut oil body proteins were successfully extracted into coconut oil body membrane extracts and were involved in the emulsification of the oil droplets by using the COBM.

5.2.2 ζ - potential

The ζ - potential of CSME and COBM emulsions at 0.8 % (w/w) protein were measured at the pH where these emulsions were prepared (without pH adjustment) (Table 5.2). The effect of pH on ζ - potential of CSME and COBM emulsions was discussed in the next

chapter. The ζ - potential of CSME emulsion (-21 mV) was lower than that of COBM emulsion (-35 mV) which may be due to the higher pH of the COBM emulsion and different proteins presenting in CSME and COBM emulsions. Nevertheless, the ζ - potential of CSME and COBM emulsions were higher than that of freshly extracted coconut milk emulsion (-16 mV) (Tangsuphoom & Coupland, 2009).

Table 5.2 ζ - potential of freshly extracted coconut milk, coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM) emulsions

| Sample | pH | ζ - potential (mV) |
|---------------------------------|-----|--------------------------|
| Freshly extracted coconut milk* | 6.2 | -16 |
| CSME emulsion | 6.3 | -21 \pm 1.0 |
| COBM emulsion | 7.9 | -35 \pm 1.6 |

*from Tangsuphoom and Coupland (2009)

5.2.3 Particle size and distribution

The particle size ($d_{4,3}$) of CSME and COBM emulsions are shown in Figure 5.2. In order to dissociate flocculated droplets and determine their flocculation status, the particle size of emulsions was measured in SDS solution (final SDS concentration in the sample was 1%, w/v).

The particle size ($d_{4,3}$) of CSME emulsion (0.2%, w/w, protein) was 14 μm in water and 12 μm in SDS solution (1% w/v). In comparison, the particle size of CSME emulsion decreased with an increase in protein concentration and obtained the smallest particle size at 0.8% (w/w) protein, which were about 10 μm and 5 μm in the absence or presence of SDS, respectively. The differences between the particle sizes with and without treated by SDS solution indicate some flocculation in samples.

The particle size of COBM emulsion slightly decreased with an increase in protein concentrations used for emulsification. The “true” droplet size of the droplets (in SDS) reduced only slightly from 1.8 μm at 0.2% (w/w) protein to about 1 μm at 0.8% (w/w) protein. At a given protein concentration, the particle size of COBM emulsion was much smaller than that of CSME emulsion. The extent of decrease in the particle size of COBM emulsion was smaller as compared to that of CSME emulsion.

Between 0.2 to 0.8 % (w/w) proteins in emulsions, the higher protein concentration in CSME emulsion induced stronger flocculation, while the COBM emulsion was largely unaffected at different protein concentrations. The particle size and flocculation results may indicate that COBM had stronger emulsifying properties than the CSME.

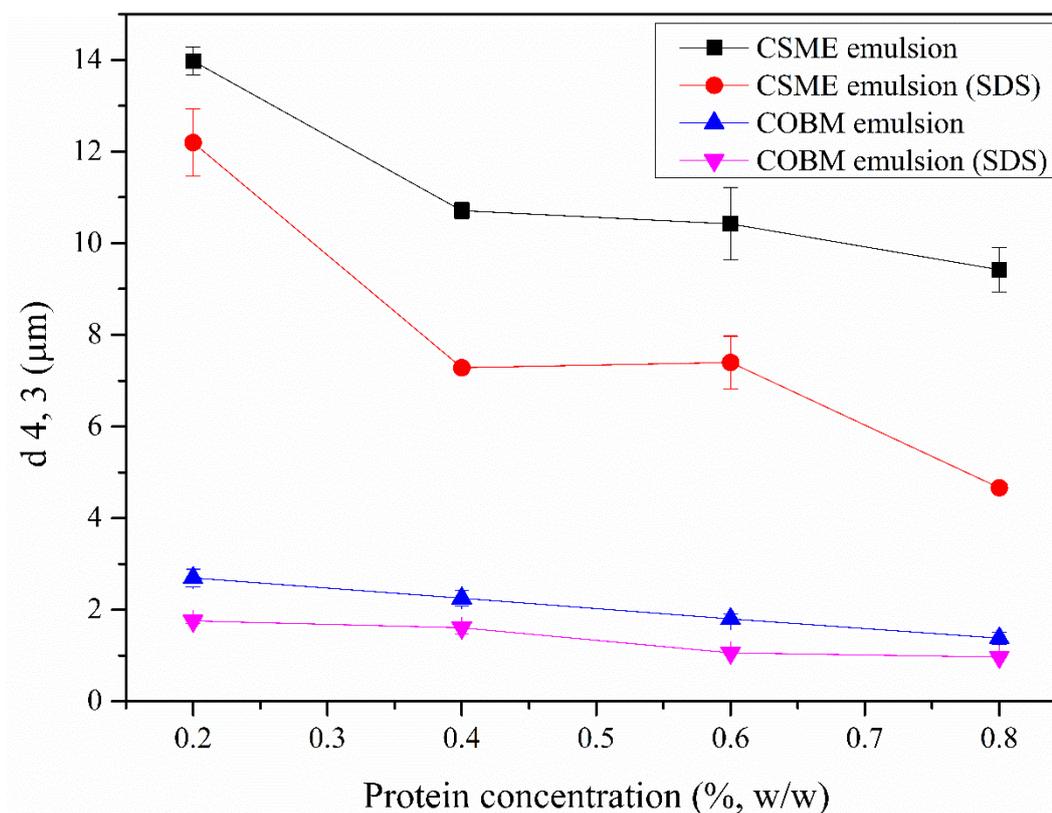


Figure 5.2 Effect of protein concentrations on particle size ($d_{4,3}$) of emulsions stabilized by coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM) and their emulsions dispersed in 1% SDS solution

The particle size distributions of CSME emulsion (at 0.4 and 0.8%, w/w, protein) were nearly similar, with 3 peaks, which consisting of a single large peak at 10 μm and 2 small peaks between 0,1 and 1 μm (Figure 5.3, A). The particle size distribution of CSME emulsion (0.4%, w/w, protein) dispersed in SDS solution was more or less similar to that dispersed in water, indicating that there were at least three populations of droplets in the CSME emulsion with very little flocculation. The largest peak of CSME emulsion (0.8%, w/w, protein) shifted left when treated with SDS solution, suggesting more flocculation happened with the increase in protein concentration.

The small peak (at around 5 μm) of COBM emulsion (0.4%, w/w, protein) without treated by SDS solution disappeared in that treated by SDS solution, indicating that there was a slight flocculation in COBM emulsion (0.4%, w/w, protein). The particle size distributions of COBM emulsion (0.8%, w/w, protein) with or without treated by SDS solution had only 1 peak (at around 1 μm). This suggests less flocculation in COBM emulsions occurred, with the increase in protein concentration.

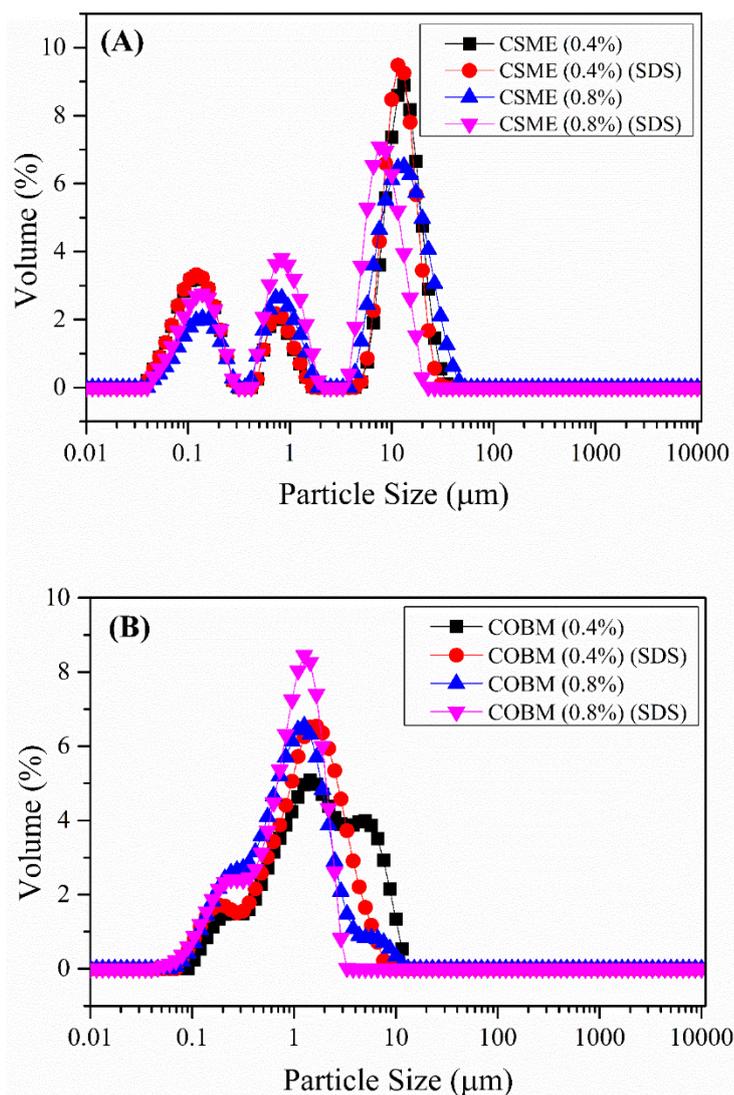


Figure 5.3 Particle size distributions of emulsions. (A) Coconut skimmed milk extract (CSME) 0.4% and 0.8% (w/w); and (B) Coconut oil body membrane (COBM) 0.4% and 0.8% (w/w). Samples were either diluted in buffer (corresponding pH values) or 1% (w/v) SDS solution

5.2.4 Emulsion stability

The extent of creaming of CSME and COBM emulsions was tested by measuring the creaming index in emulsions stored undisturbed at 20 °C (Figure 5.4). Under the influence of gravity, the emulsion droplets separated into a transparent clear bottom layer and an opaque cream layer during storage (Figure 5.5).

CSME emulsion (0.4% w/w, protein) exhibited the highest phase separation (73% creaming index) in the shortest time (6 hours). When the protein concentration was doubled to 0.8 % (w/w), the creaming index nearly halved to 45% and it took a longer time for phase separation (>10 hours). These results indicate that the increase in protein concentration decreased the particle size of droplets and these smaller droplets had a higher stability against phase separation. Figure 5.5 shows the extent of phase separation in CSME emulsions after 36 h. In comparison, the COBM emulsions were stable to creaming even after 36 h irrespective of the protein concentration (0.4 or 0.8% w/w, protein). This indicated that COBM emulsion at 0.4 % protein concentration was enough to keep emulsions stable against creaming.

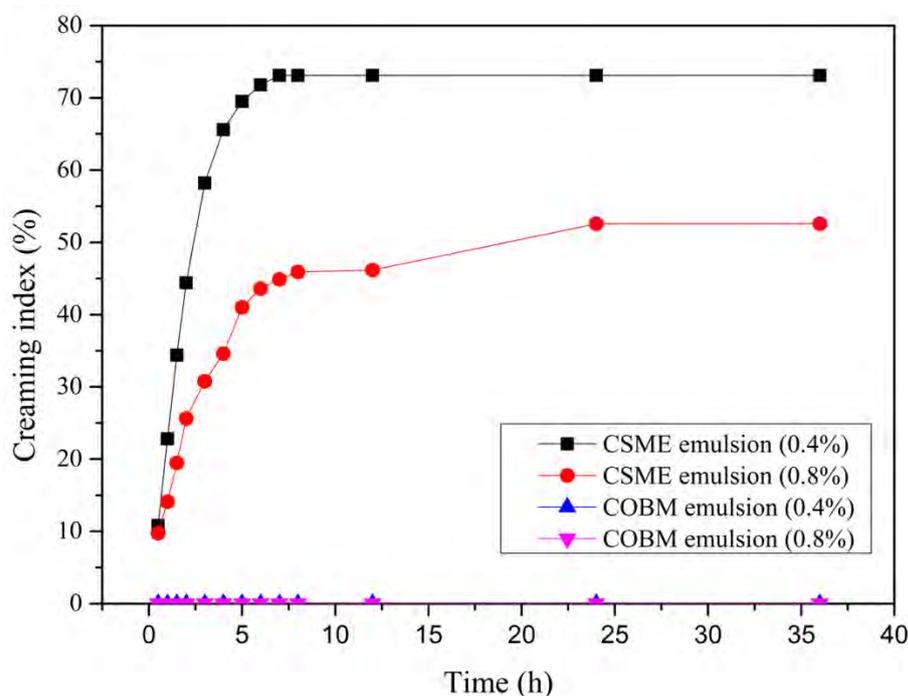


Figure 5.4 Creaming indices of coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM) formed emulsions at two protein concentrations (0.4 and 0.8%, w/w) respectively

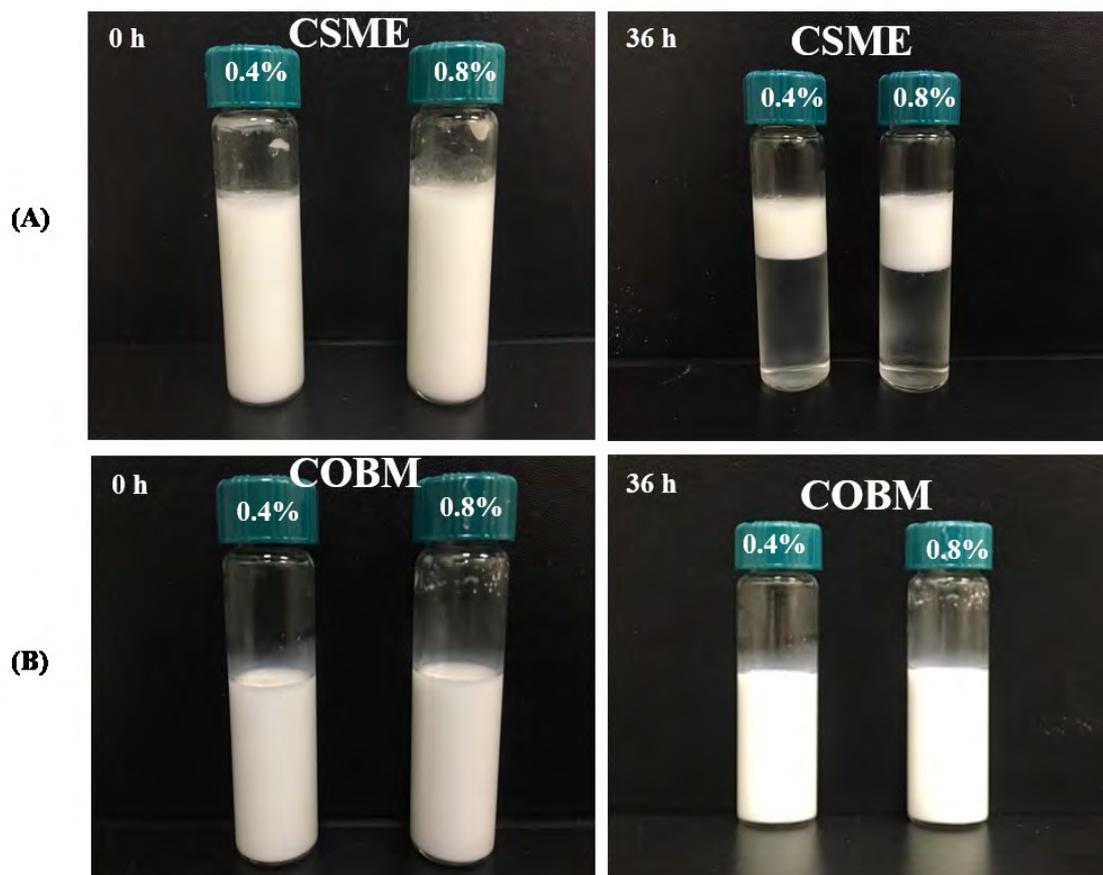


Figure 5.5 Images showing stability of (A) coconut skin milk extracts (CSME), and (B) coconut oil body membrane (COBM) formed emulsions at two protein concentrations (0.4 and 0.8%, w/w) at time 0h and 36h

The stability of CSME and COBM emulsions against long-time storage (up to 30 days, at 4 °C) were determined by measuring the particle size ($d_{4,3}$) of emulsions treated by SDS solution (Figure 5.6). The particle size of CSME emulsion (0.4%, w/w, protein) was stable in first 5 days, followed by increasing steadily during day 5 to 20. After day 20, the particle size increased significantly and showed oiling-off. For CSME emulsion (0.8%, w/w, protein), the particle size was stable in first 20 days and increased suddenly after day 25 (oiling-off). However, the particle sizes of COBM emulsion (0.4 and 0.8%, w/w, protein) were constantly in all 30 days.

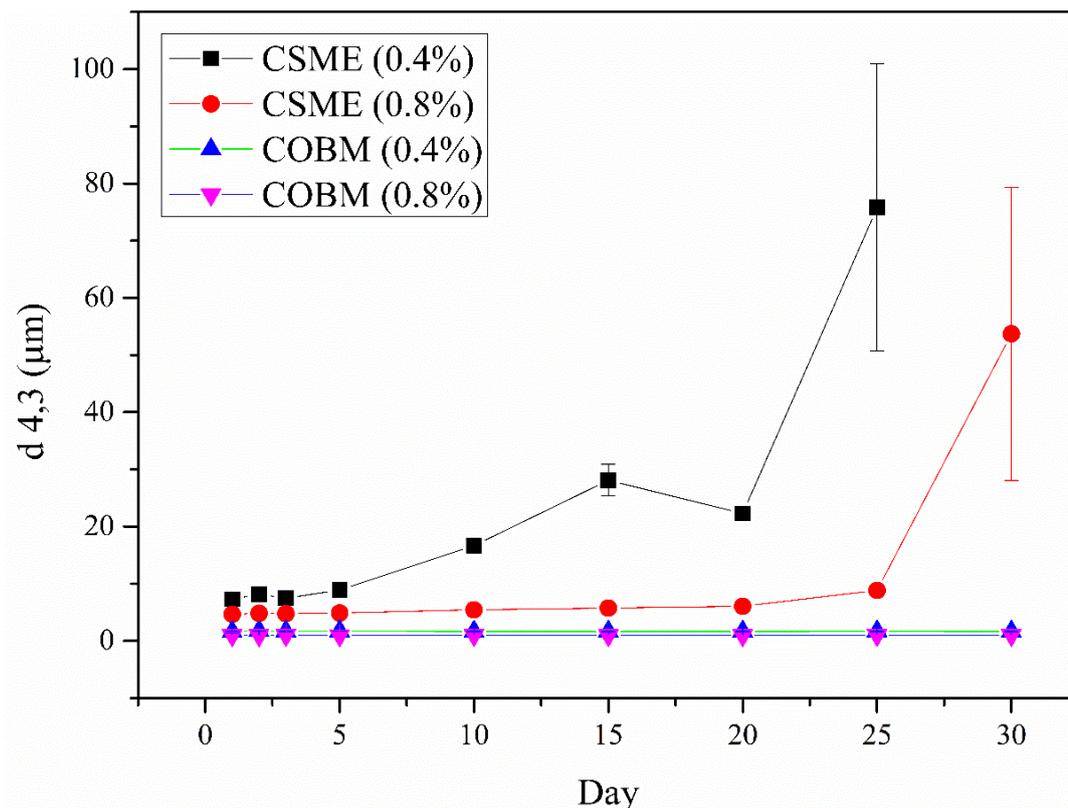


Figure 5.6 Effect of storage on the particle size ($d_{4,3}$) of coconut skim milk extracts (CSME) and coconut oil body membrane (COBM) formed emulsions (0.4 and 0.8%, w/w, protein)

5.2.5 Structure of CSME and COBM emulsion droplets membrane

The microstructure of CSME and COBM emulsions was investigated using confocal laser scanning microscopy. Emulsions (0.2 to 0.8%, w/w, protein) were stained by dyes Nile Red specific for neutral lipids, Fast Green FCF specific for proteins and LissamineTM rhodamine B specific for phospholipids. The oil droplets consisted of a large intense red fluorescent core region exhibited by Nile Red when present in the organic phase (Greenspan, Mayer, & Fowler, 1985), representing the neutral triacylglycerols (TAG) of soybean oil. The TAG core was encompassed in a thin layer of proteins represented by the green fluorescence of the dye Fast Green FCF. This dye binds electrostatically bound with protein producing a green fluorescent protein complex. The LissamineTM rhodamine B (Rd-DHPE) dye could bind with phospholipids presenting on the surface of the droplet and showed intense red fluorescence.

The CLSM images of the CSME and COBM emulsions stained by Nile Red and Fast Green FCF are shown in Figure 5.7 and Figure 5.8, respectively. From Figure 5.7 (image A-D), with the increasing of protein concentrations, the majority diameter of oil droplets decreased from approximately 15 μm (0.2%, w/w, protein) to 4 μm (0.8%, w/w, protein), which was in agreement with the particle size data (Section 5.3.3). Slight flocculation could be observed in CSME emulsion (0.8%, w/w, protein) (Figure 5.7, image D). From Figure 5.8 (image A-D), at the given protein concentrations (0.2 to 0.8 %, w/w), the majority diameters of oil droplets retained below 2 μm , which was in agreement with the particle size data (Section 5.3.3). No obvious flocculation was observed at all protein concentration.

In Figure 5.7 (image E) and Figure 5.8 (image E), LissamineTM rhodamine B (Rd-DHPE) dye could bind with the phospholipids presenting on both droplet surfaces of CSME and COBM emulsions and showed intense red fluorescence. Similarly, when CSME and COBM emulsions (0.8%, w/w, protein) were stained by Fast Green FCF (Figure 5.7 and Figure 5.8, image F), the green fluorescence, presenting on both droplets surfaces of CSME and COBM emulsions. These results indicate that CSME and COBM emulsions droplets surface consisted of both phospholipids and proteins.

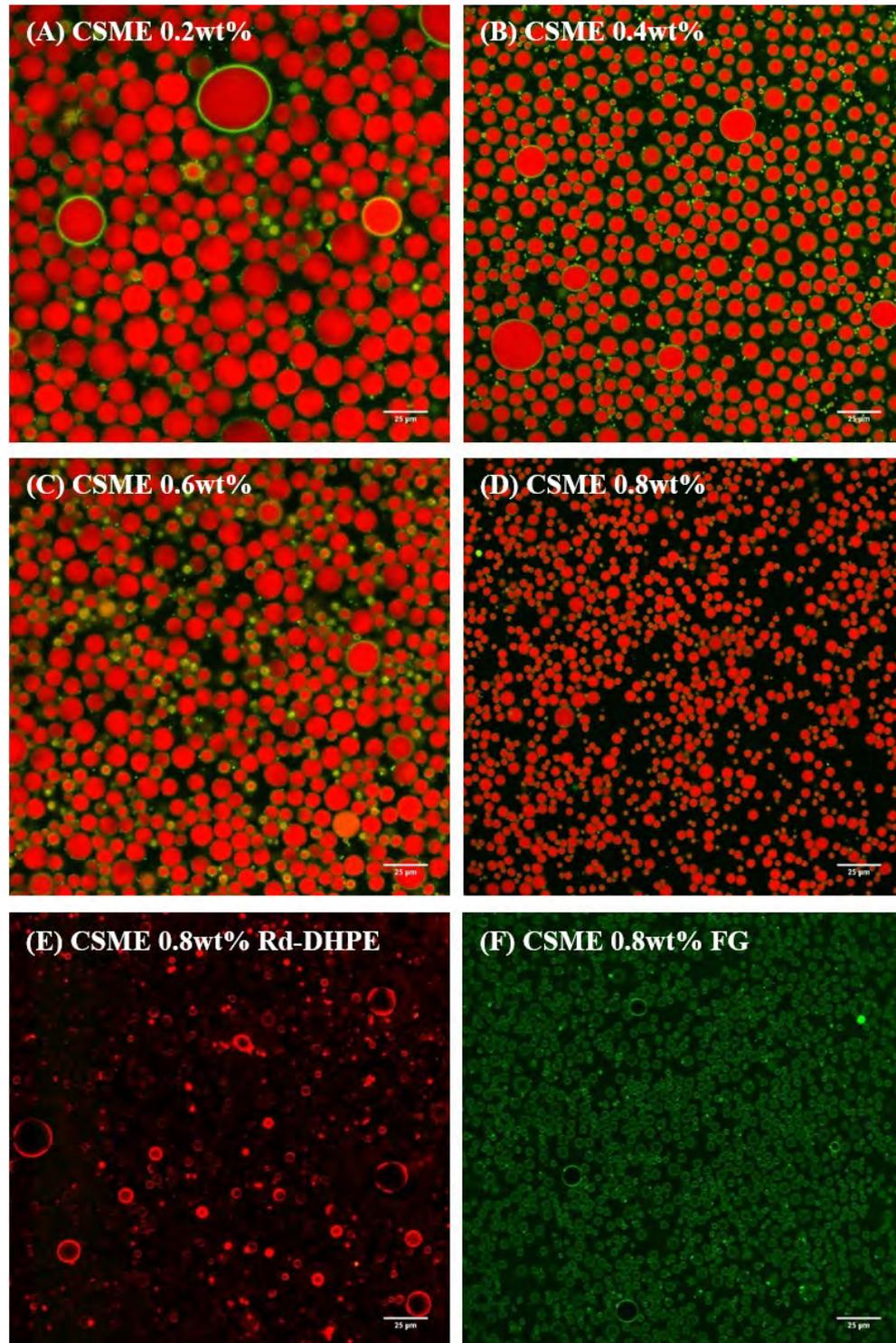


Figure 5.7 Confocal laser scanning microscopy images of coconut skimmed milk extracts (CSME) formed at 0.2 to 0.8% (w/w) protein concentrations. Droplets in A-D stained using Nile Red (neutral lipids) and Fast Green (proteins). Those in D are stained by LissamineTM rhodamine B (Rd-DHPE) showing phospholipids. F shows the separated green channels (showing protein) for CSME emulsions at (0.8% w/w). Scale bar = 25 μm

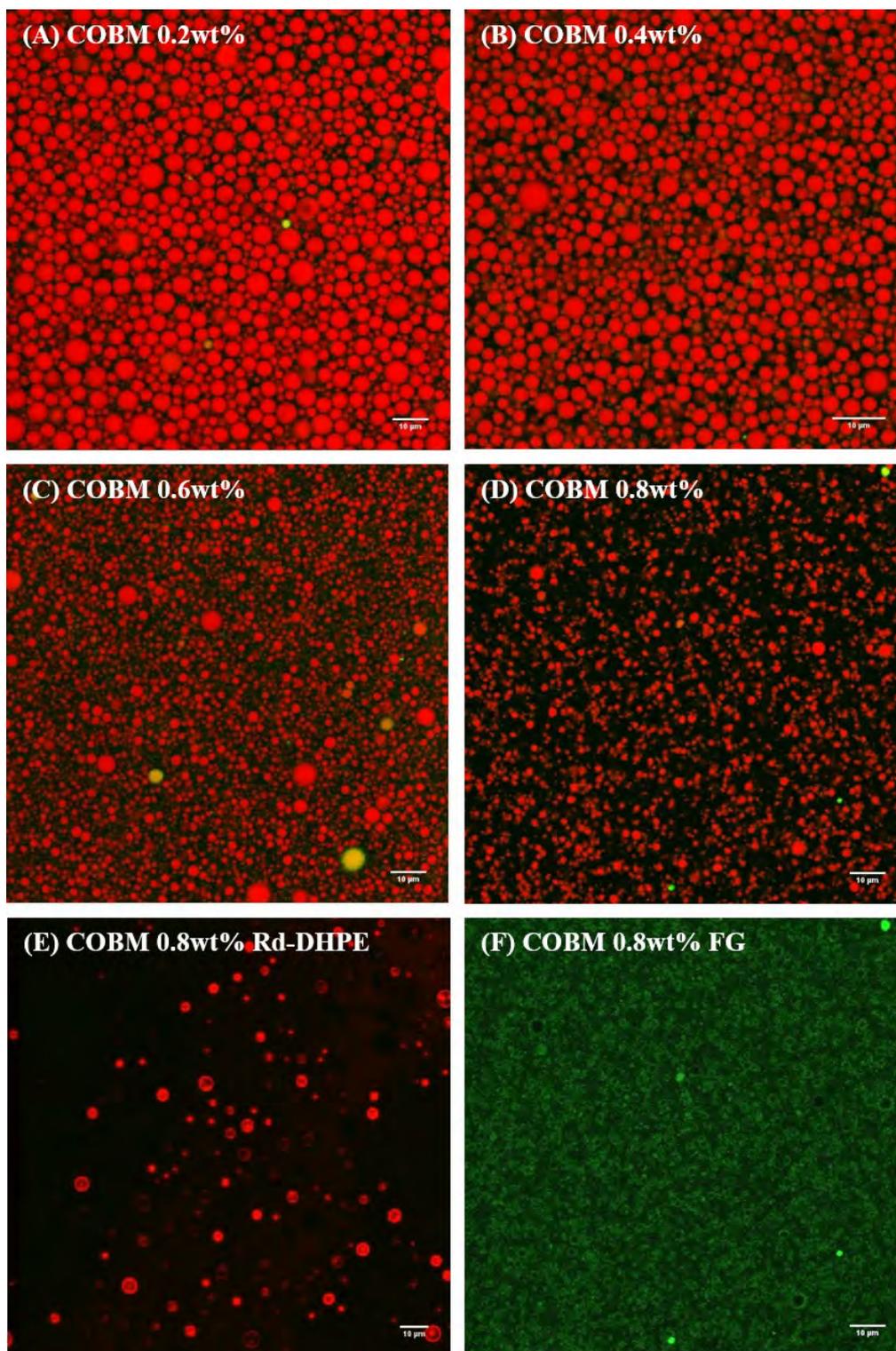


Figure 5.8 Confocal laser scanning microscopy images of coconut skimmed milk extracts (COBM) formed at 0.2 to 0.8% (w/w) protein concentrations. Droplets in A-D stained using Nile Red (neutral lipids) and Fast Green (proteins). Those in D are stained by LissamineTM rhodamine B (Rd-DHPE) showing phospholipids. F shows the separated green channels (showing protein) for COBM emulsions at (0.8% w/w). Scale bar = 10 µm

Enzymatic studies to probe the structure of emulsions

To determine the interfacial structure of emulsions, the coconut skimmed milk extract (CSME) and coconut oil body membrane (COBM) stabilized emulsions (0.8%, w/w protein) were selected since CSME emulsion performed better stability at this protein concentration (mentioned in Section 5.3.4). The emulsions were treated separately by pepsin, trypsin and phospholipase A₂, and the effect of enzyme treatments on the particle size ($d_{4,3}$) was measured after dispersing the treated emulsions in 1% SDS solution (Figure 5.9).

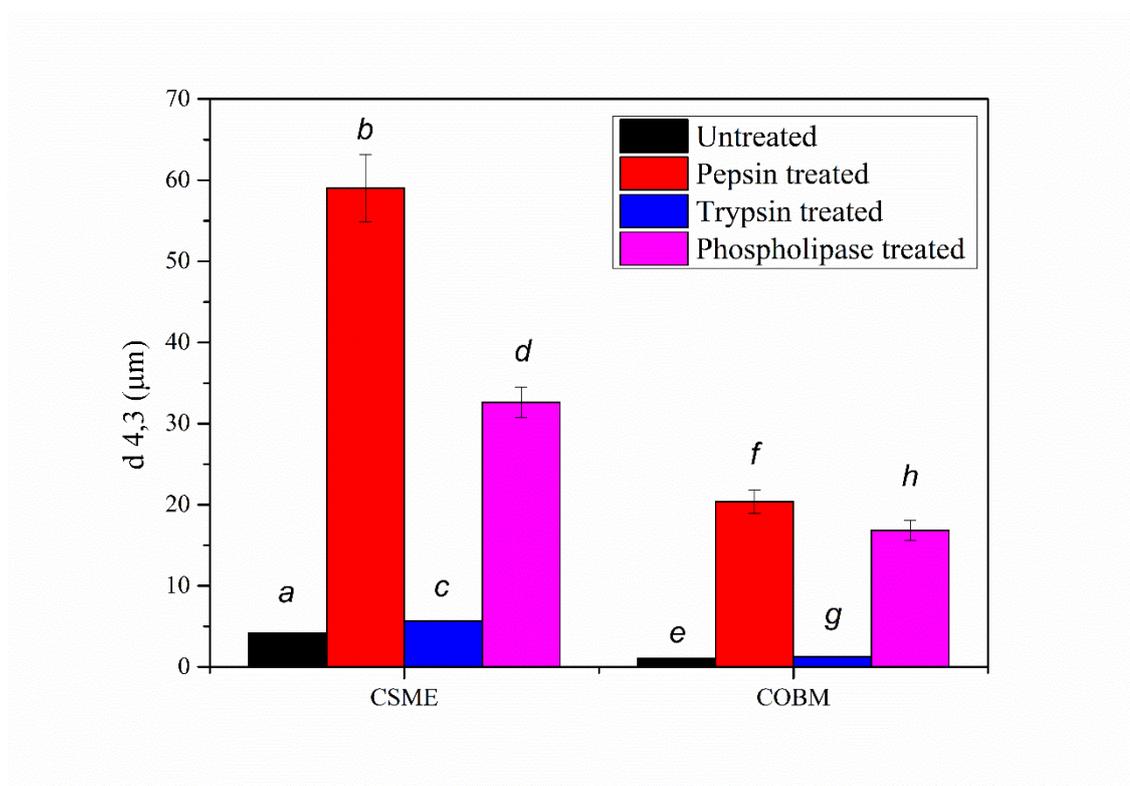


Figure 5.9 Particle size ($d_{4,3}$) of coconut skimmed milk extracts (CSME) formed emulsion and coconut oil body membrane (COBM) formed emulsion treated by pepsin, trypsin and phospholipase A₂, separately, dispersed in 1 % SDS solution. Different lower case letters represent significant differences ($p < 0.05$) between samples

The particle size ($d_{4,3}$) of the CSME emulsion prior to enzyme treatment was 4.2 μm , which increased significantly upon treatment with pepsin and phospholipase A₂, reaching

to 59 and 32 μm , respectively. In comparison, the particle size of the emulsions was more or less similar (5.6 μm) upon treatment with trypsin. The particle size ($d_{4,3}$) of COBM emulsion showed the same trend as CSME emulsion. The particle size of COBM emulsion increased to 20.3 and 16.8 μm after pepsin and phospholipase treatment, respectively, but remained largely unaffected (1.3 μm) after trypsin treatment. It is interesting to note that the pepsin treatment affected the particle size of CSME and COBM emulsions almost by an equal extent, while the COBM emulsion was more sensitive to destabilization by phospholipase treatment than CSME emulsion. These results may suggest that both proteins and phospholipids were responsible for the stabilization of CSME and COBM emulsions.

The confocal laser scanning microscopy images of CSME and COBM emulsions (Figure 5.10) showed an increase in the droplet size, which supports the particle size data. Compared with untreated CSME and COBM emulsions, both pepsin and phospholipase treated CSME and COBM emulsions showed larger oil droplets, while the trypsin treated CSME and COBM emulsions remained constant. The different extent of flocculation in the phospholipase treated CSME and COBM emulsions may be induced by different surface composition and the CaCl_2 in the buffer used for the PLA_2 digestion.

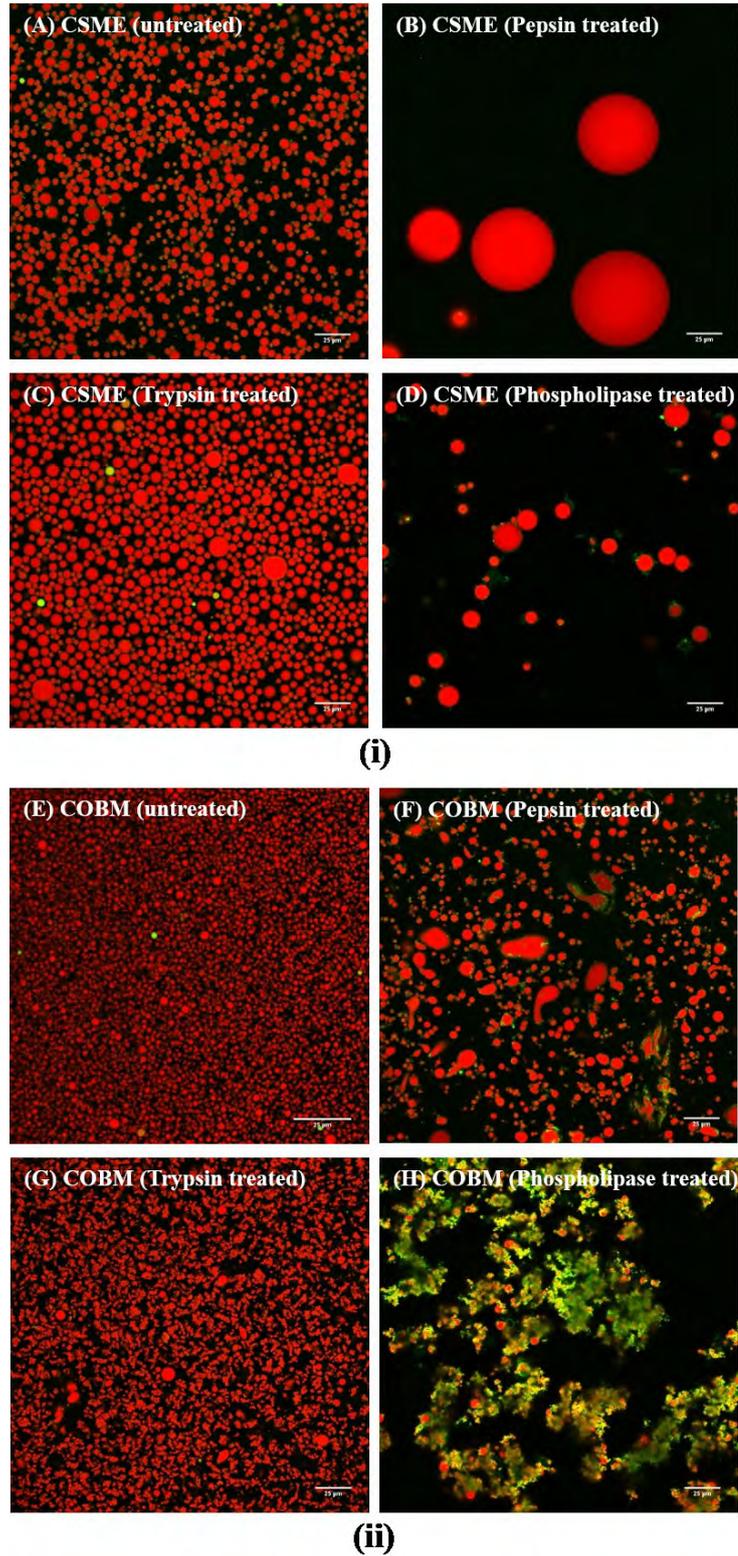


Figure 5.10 Confocal laser scanning microscopy images of (i): coconut skimmed milk extracts (CSME) formed emulsion (ii): coconut oil body membrane (COBM) formed emulsion treated by pepsin, trypsin and phospholipase A₂. The droplets were stained by Nile Red and Fast Green FCF (Scale bar = 25 µm)

To determine the composition of interfacial proteins in the enzyme-treated oil droplets, the enzyme treated CSME and COBM emulsions were centrifuged at 17000 *g* for 10 min to get a cream layer. The surface proteins were then extracted using the method described by Tzen and Huang (1992) and analysed by SDS PAGE (Figure 5.11). It must be noted here that the CSME emulsion showed oiling off after pepsin treatment and no cream layer was obtained after centrifugation. Therefore, for the CSME emulsion, the uncentrifuged pepsin-treated emulsion (instead of surface protein extracts) was used as the sample for SDS PAGE.

As discussed in the above section, the interfacial proteins in the CSME emulsion had molecular weights ranging from 10 kDa to 75 kDa. The most prominent band present at the interface in untreated emulsions was that corresponding to an approximate molecular weight of 50 kDa (band A) (Figure 5.11, i). In comparison, distinct differences in the bands could be identified when the droplets were treated with pepsin. Most notable was the absence of the bands in Region R1. Instead, there were numerous bands < 20 kDa (Region R2). In comparison, the trypsin-treated CSME emulsion showed an intact band at 50 kDa (band B) and more or less similar polypeptide bands as that in untreated CSME emulsion.

When it comes to COBM emulsion, there were 3 major bands at around 55, 37 and 15 kDa (band C, D and E) in the surface protein of untreated emulsion, which attended in trypsin treated COBM emulsion (band F, G and H) (Figure 5.11, ii). However, these 3 bands, especially band C (55 kDa) and band D (37 kDa), were absented in pepsin treated COBM emulsion (Region R3). Instead, there were numerous bands < 35 kDa (Region R4).

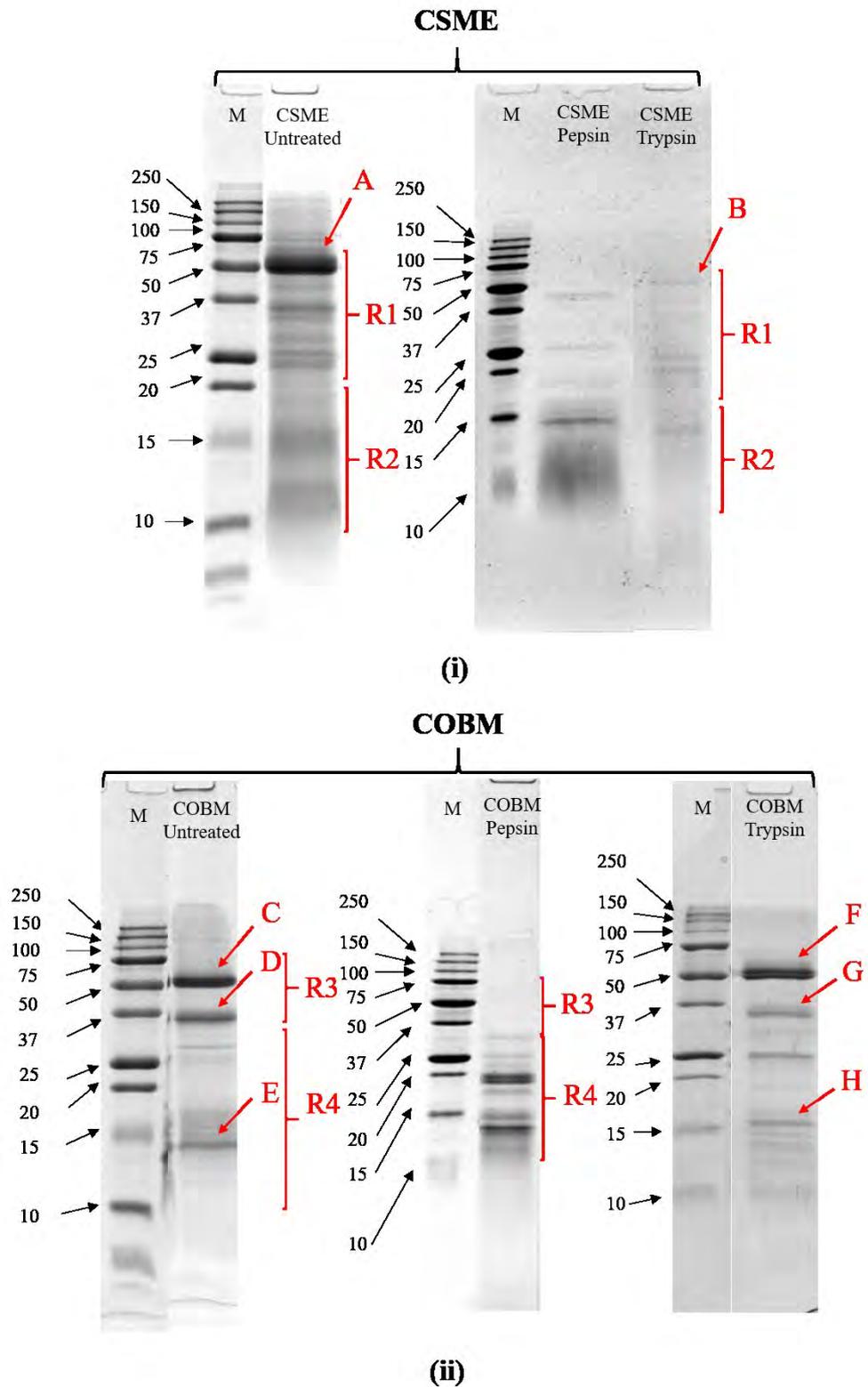


Figure 5.11 SDS-PAGE of interfacial proteins in (i) CSME and (ii) COBM emulsions after treatments with proteases pepsin and trypsin. M refers to marker. The pepsin-treated CSME emulsion showed oil separation hence the aqueous phase was used for the SDS-PAGE analysis

5.3 Discussion

The particle size of CSME and COBM emulsions suggests that at a given protein concentration, COBM could form much smaller emulsion particle size and had the better emulsifying capacity. This could be attributed to the different types of proteins and the concentration of phospholipids contained in these extracts. These proteins in the respective extracts may have different adsorption rate, surface activities and/or surface loads which may have an effect on their emulsifying properties (McClements, 2015; McClements et al., 2017).

For both CSME and COBM emulsions (0.4%, w/w, protein), most of the protein adsorbed on emulsion droplets. Doubling the protein concentration to 0.8% (w/w) resulted in smaller droplet size and the protein content on droplet surface increased more than double. The possible reason for this could be that the higher protein concentration could help to create small emulsion droplets and facilitate the covering a larger newly formed surface area (McClements, 2015).

The effect of protein concentration on particle size was more pronounced in CSME emulsion than COBM emulsion. The particle size of CSME emulsion decreased with an increase in protein concentration, while the COBM emulsion was largely independent of protein concentration. During emulsification, the droplet size is limited by the concentration of emulsifiers available to cover the freshly created surface area of newly formed droplets (Tcholakova, Denkov, Ivanov, & Campbell, 2002). In comparison, if the emulsifier's concentration exceeds a critical concentration or is more surface active, the newly created surface area would be covered by the emulsifier and the residual emulsifier may remain in the aqueous phase. In this case, the droplet size would mainly depend on the input energy for emulsification and not emulsifiers concentration (McClements, 2004; Ye et al., 2013). Thus, for CSME emulsion, the droplet size was limited by the amount of protein available for stabilization at the interface. The droplet size of CSME emulsion reduced steeply by increasing the CSME, which may indicate that the droplet size is limited by the maximum surface area that CSME could cover. CSME protein concentration up to 0.8 % (w/w) was insufficient to fully cover the surface area during homogenization. In contrast, for the COBM emulsion, there was no appreciable reduction of the particle size of COBE emulsion, especially from 0.6 to 0.8 % of protein, which may indicate that 0.6 % of the protein was enough to cover the droplets. The droplet

formation was probably governed by the emulsification step irrespective of the protein concentration used in emulsification.

From the particle size distribution, CSME emulsion always had 3 populations of droplets with “true” particle size, which suggests that there were not enough protein in CSME to fully cover the droplets, so the CSME emulsion was formed as 3 populations peaks. When doubling the protein concentration from 0.4 to 0.8% (w/w), the biggest population peak decreased, while the small population peak at 1 μm increased. This result suggests that increased protein concentration in CSME emulsions provided more protein to cover the droplet surface and CSME emulsions had a tendency to form small droplets. In comparison, COBM emulsion had only 1 population of droplets with “true” particle size, indicating that there were sufficient proteins to decrease particle size and form uniform size emulsions.

The CSME emulsion showed a different extent of flocculation at all protein concentrations. This may indicate that proteins in CSME emulsion may not enough to fully cover the droplets (as mentioned above), which may lead to bridge flocculation. However, the proteins in COBM emulsion was sufficient to cover the droplets and probably did not require sharing of protein molecules amongst different droplets which inhibited the flocculation in COBM emulsion (Ye et al., 2013).

Another reason for some extent of flocculation observed in CSME emulsion may be the relatively weak repulsive electrostatic force between the droplets of CSME emulsion (McClements, 2004). Generally, emulsions could be stable to against aggregation when their ζ - potential are higher than ± 30 mV (Riddick, 1968). The COBM emulsion had higher ζ - potential (above -35 mV), indicating that COBM emulsion could create stronger electrostatic repulsive to avoid flocculation, while the ζ - potential (above -21 mV) of CSME emulsion was relatively low, indicating that their electrostatic repulsive may not efficient enough to completely avoid the droplets to come in contact with each other.

The large droplet size in the CSME emulsion may increase the tendency to phase separate. The flocculation in CSME emulsion could also enhance the rate of creaming. (Iwanaga et al., 2007; McClements, 2015). During storage at 4 °C, CSME emulsion showed

coalescence. With the increase in protein concentration (from 0.4 to 0.8 %, w/w), the coalescence was delayed (from day 5 to day 20), indicating that the increase in protein loaded on droplet surface may protect the integrity of droplets. However, the protein concentration (up to 0.8 %, w/w) was insufficient to stabilize CSME emulsion for long-term storage. In comparison, the COBM emulsion showed remarkable stability against creaming and was stable even after 30 days at 4 °C. The stability of COBM emulsion droplets may be attributed to the high electrostatic repulsion (above +35 mV) or steric hindrance provided by surface protein. Thus, CSME and COBM emulsions exhibited different physicochemical properties, which may be attributed to the different protein compositions at their interface.

The SDS-PAGE showed distinct differences in the surface protein compositions of CSME and COBM emulsions. The major protein in both CSME and COBM emulsions was around 55 kDa, which corresponds to the report of the major protein molecule weight in coconut meat from other research (Patil & Benjakul, 2017). In CSME emulsion, a distinct band at around 50 kDa (band A, Figure 5.1) was only present in the droplet phase but not in the serum, indicating that proteins in this band most likely participated in emulsion formation and stabilization. Several other protein bands were also noted on the CSME emulsion droplets although at a much lower intensity. Different from CSME emulsion, in COBM emulsion, the 3 most prominent surface proteins (around 55, 37 and 15 kDa) also presented in its serum phase which may indicate that these 3 proteins had a higher tendency to adsorb on oil droplets but did not 100 % adsorb on the surface.

The molecular weight of oleosins found in oilseeds was at a range of 15 to 30 kDa (Tzen & Huang, 1992; Yamaguchi, 2010), which is corresponded to the molecular weight of surface proteins of COBM emulsion. The major bands (around 15 and 50 kDa) found in coconut oil bodies (Dave et al., 2019) were also noted in the surface protein composition of COBM emulsion. This indicates that the interfacial protein composition of COBM emulsion may be similar to that of natural coconut oil body.

The structures of CSME and COBM emulsions were investigated by perturbing the interface using different enzymes pepsin, trypsin and phospholipase A₂ (PLA₂). Both CSME and COBM emulsions were destabilized by pepsin to a similar extent, indicating

that the surface protein composition of both emulsions had sequences with aromatic and hydrophobic residues on the surface, which could be acted upon by pepsin (Fruton, 2002).

In comparison, the protease trypsin did not affect the particle size of both CSME and COBM emulsions. The oleosin proteins are highly hydrophobic and consist of only few positively charged residues for the action of trypsin (Dave et al., 2019). Thus, the higher stability of the COBM emulsion against destabilization by trypsin is not unexpected. It is not known why the CSME emulsion remained stable upon trypsin treatment. It is possible that the positively charged groups were shielded from the solvent. It is also possible that the surface of CSME emulsion contained fewer positively charged residues on the surface.

The COBM emulsion was more sensitive to the PLA₂ treatment than CSME emulsion, which indicates that more phospholipids participated in droplet stabilization in COBM emulsion. Taken together with particle size data (size of COBM emulsion < CSME emulsion) these results may suggest that COBM had higher phospholipids content. The higher inorganic phosphorous content in the CSME may have been contributed by the contaminant inorganic phosphorous from water or from coconut milk itself.

The structure of freshly oil bodies consists of a triglyceride core surrounded by an interfacial layer consisting of phospholipids and oleosins (Dave et al., 2019; Tzen & Huang, 1992). The oleosins have been known to protect the phospholipid layer by blocking the access of the enzyme PLA₂ to the phospholipid layer by steric interactions (Tzen & Huang, 1992). The droplets of CSME and COBM emulsions showed that both proteins and phospholipids present at the interface. The enzymatic study revealed that the arrangement of protein and phospholipids on the surface was probably similar in both emulsions but different than the conventional structure of oil bodies.

5.4 Conclusion

In conclusion, the coconut oil body membrane (COBM) is more effective emulsifier than coconut skimmed milk extract (CSME). The emulsions formed by COBM had smaller droplet size and were more stable during storage than CSME emulsion. With the increase in protein concentrations (0.2 to 0.8%, w/w), the particle size of CSME emulsion was decreased, while there was not too much influence on COBM emulsion, which also

suggests that COBM is a more effective emulsifier. The structural design of the emulsion droplets in the two emulsions was similar with both phospholipids and proteins present at the interface. The enzymatic studies provided insights into the behaviour of emulsion droplets during gastrointestinal digestion.

6. Effect of pH and ionic strength on the emulsions stabilized by coconut oil body membrane (COBM) or coconut skimmed milk extract (CSME)

6.1 Introduction

In the previous chapter, the physicochemical characteristics of the emulsions stabilized by coconut oil body membrane (COBM) or coconut skimmed milk extract (CSME) was investigated under the un-adjusted pH conditions. The changes in aqueous conditions such as pH or ionic strength have been reported to have a significant effect on the properties of the emulsion. Therefore, the objective of the study in this chapter is to investigate the effect of pH and ionic strength on the stability of CSME and COBM emulsions. Investigation of the behaviours of these emulsions at different pH and ionic strengths may allow prediction of their stability under diverse conditions such as gastrointestinal digestion or in complex food formulations.

6.2 Results

6.2.1 Effect of pH on emulsions

CSME emulsions at 0.4 and 0.8%, w/w, protein (CSME1 and CSME2 emulsions, respectively) and COBM emulsions at 0.4 and 0.8%, w/w, protein (COBM1 and COBM2 emulsions, respectively) were prepared as the protocol described in Section 3.2.2 of Chapter 3. These emulsions were adjusted to different pH (2 to 8). The pH of the CSME and COBM emulsions at formation (without pH adjustment) was about pH 6.4 and 7.8, respectively. At their unadjusted pH values, the ζ - potential of CSME and COBM emulsions were -22 mV and -38 mV, respectively. The pH dependence of the ζ - potential for CSME and COBM emulsions is illustrated in Figure 6.1.

For CSME emulsions, an increase in pH ($\text{pH} > 6.4$), the emulsion droplets became increasingly negatively charged and their ζ - potential reached a maximum (approximately -30 mV) at pH 8. The isoelectric pH of the droplets at which the ζ - potential became 0 mV was approximately pH 4.5. Below their isoelectric point, the decrease in pH resulted in a high positive charge on droplets, which reached a maximum (approximately +35 mV) at pH 2.

The ζ - potential of COBM emulsions showed a similar trend as that of CSME emulsions. The ζ - potential of COBM emulsions became 0 between pH 4.5 and 5.0 (approximately

4.7). Below the pI, the droplets had high positive charge and the ζ - potential reached the maximum value (+27 mV) at pH 2. When pH > pI, the droplets became increasingly negatively charged and the ζ - potential of COBM emulsions reached the maximum value (above -35 mV) at pH 8. Between pH 4 and 6, the ζ - potential of COBM emulsions were more sensitive to changes in pH as compared to the CSME emulsions. In general, the ζ - potential of COBM emulsions was higher than that of CSME emulsions at nearly all pH values investigated.

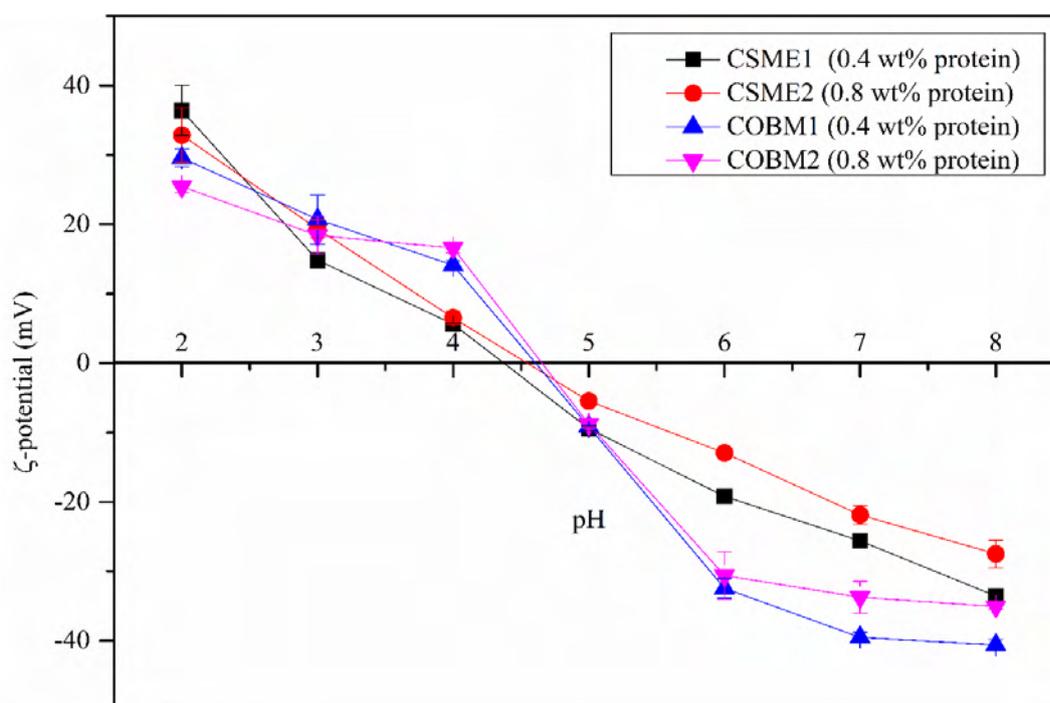


Figure 6.1 ζ - potential of coconut skimmed milk extracts (CSME) emulsions and coconut oil body membrane extracts (COBM) emulsions at two protein concentrations (0.4 and 0.8%, w/w) at different pH (2-8)

The $d_{4,3}$ values of CSME1 and CSME2 emulsions (0.4 and 0.8% w/w, protein, respectively) at different pH are shown in Figure 6.2. Between pH 5 to 8, the $d_{4,3}$ values of CSME1 and CSME2 emulsions (with or without treated by SDS solution) remained relatively constant at approximately 8 and 4.5 μm , respectively. With the decrease in pH, especially below pH 5, the $d_{4,3}$ values of CSME1 and CSME2 emulsions (without SDS solution treatment) increased significantly and reached about 20 μm for CSME1 and

above 60 μm for CSME2, respectively. It is interesting to note that the particle size of CSME1 and CSME2 emulsions dispersed in SDS solution were close to the particle size of CSME1 and CSME2 emulsions without SDS solution treatment, which indicated that coalescence dominated the increase in the particle size.

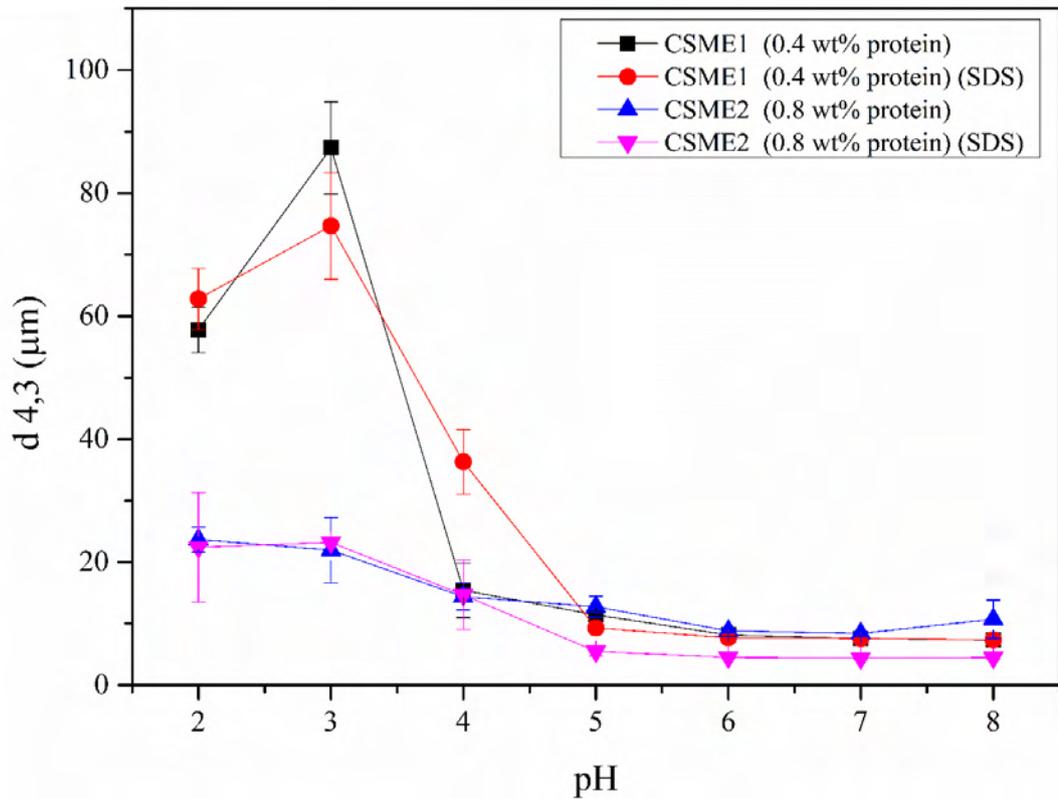


Figure 6.2 Particle size ($d_{4,3}$) of coconut skimmed milk extracts (CSME) emulsions at two protein concentrations (0.4 and 0.8%, w/w) at different pH (2-8) and their emulsions in 1% SDS solution

The particle size ($d_{4,3}$) of COBM1 and COBM2 emulsions (0.4 and 0.8% w/w, protein, respectively) at different pH are shown in Figure 6.3. At all pH values studied (2 to 8), the mean droplet size of COBM1 and COBM2 emulsions dispersed in SDS solution (1% w/v) solution remained relatively unaffected at about 2 μm and 1 μm , respectively. The particle sizes of COBM1 and COBM2 emulsions (without SDS solution treatment) were stable at pH 7 and 8 (2.2 μm for COBM1 emulsion and 1.4 μm for COBM2 emulsion), followed by a moderate increase to approximately 6 μm and 4 μm respectively, when the

pH lowered to 6. The biggest particle sizes were at pH 5 (20 and 22.5 μm , respectively) which was close to the isoelectric point of the COBM emulsions (Figure 6.1). With a further decrease in pH from 5 to 2, the $d_{4,3}$ decreased to around 5 μm .

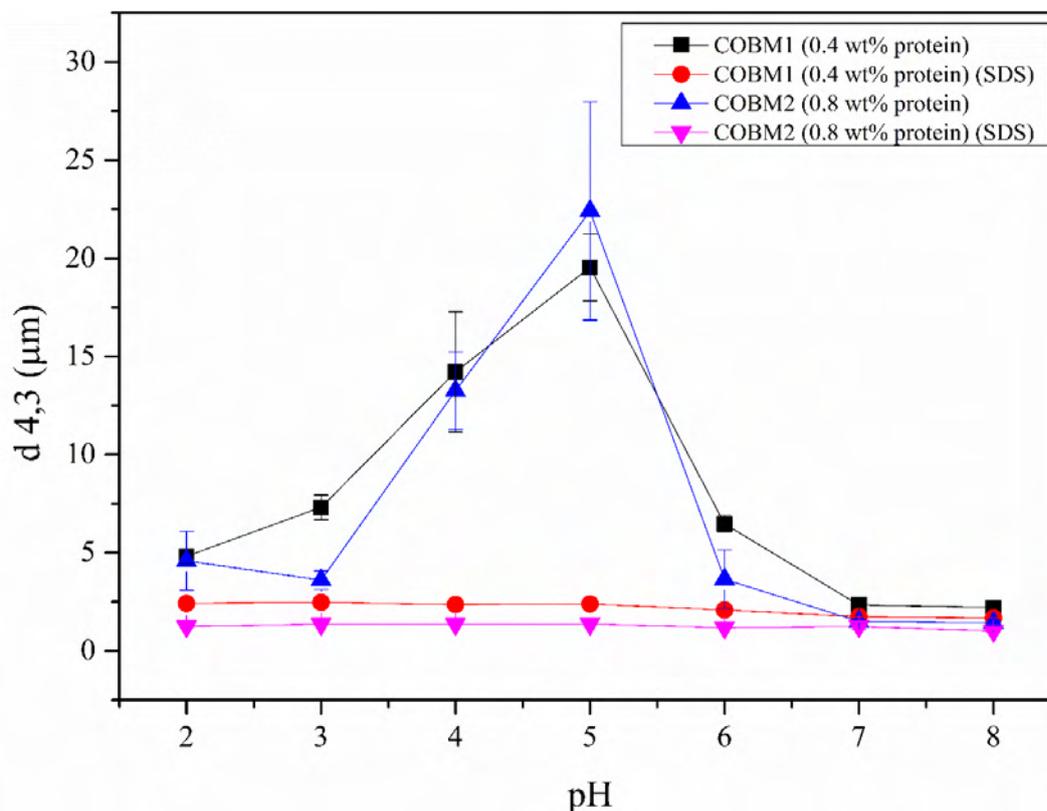


Figure 6.3 Particle size ($d_{4,3}$) of coconut oil body membrane extracts (COBM) emulsions at two protein concentrations (0.4 and 0.8%, w/w) at different pH (2-8) and their emulsions in 1% SDS solution

The microstructure of the droplets was observed by confocal laser scanning microscopy (CLSM). The CLSM images of CSME1 and CSME2 emulsions (0.4 and 0.8%, w/w, protein, respectively) at different pH (pH 2 to 8) are shown in Figure 6.4 and 6.5. The CSME emulsions were relatively stable at pH > pH 6 and the droplets remained unflocculated. The majority droplets in CSME1 and CSME2 emulsions were approximately 8 and 4.5 μm , respectively. At pH 5, the droplets were slightly flocculated, while at pH 4 and below, the size of droplets remarkably increased (above 20 μm), indicating that CSME emulsion was highly unstable at low pH (5 to 2) and coalescence was predominant. According to the particle size data, CSME1 emulsion contained larger droplets than CSME2 emulsion. However, in the CLSM images of CSME1 emulsion at pH 2 and 3,

droplets with diameters above 60 μm were absent. It is possible that the larger droplets existed in the CLSM sample but is more susceptible to creaming and may have migrated out from the plane of imaging to the surface. Another possible reason is that CSME emulsions were oiling off during the pH adjustment and only small droplets may have been sampled for imaging.

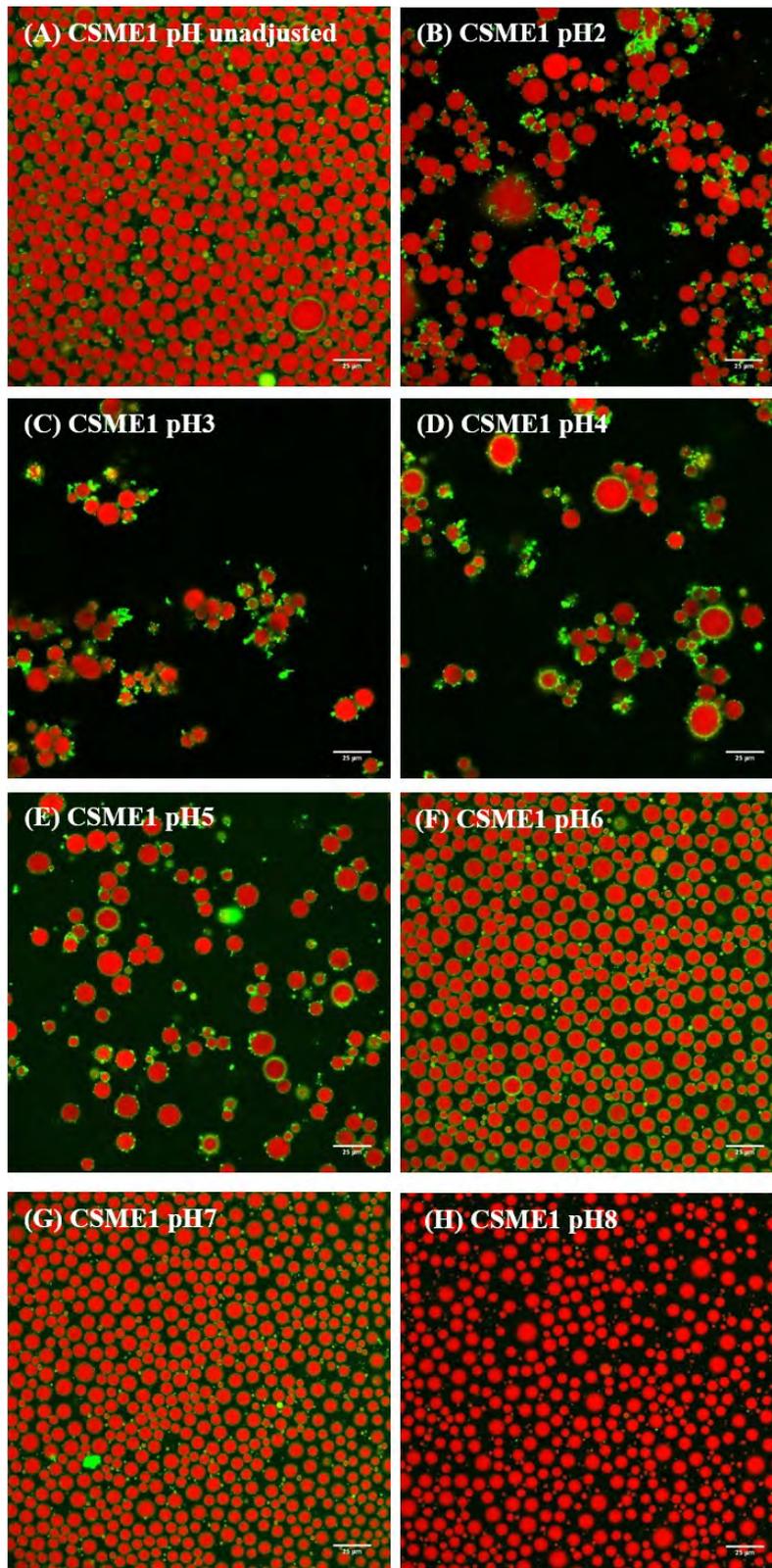


Figure 6.4 CLSM images of coconut skimmed milk extracts formed emulsion (CSME1 at 0.4%, w/w, protein) stained by Nile Red and Fast Green at different pH conditions (pH 2 - 8) (Scale bar = 25 μ m)

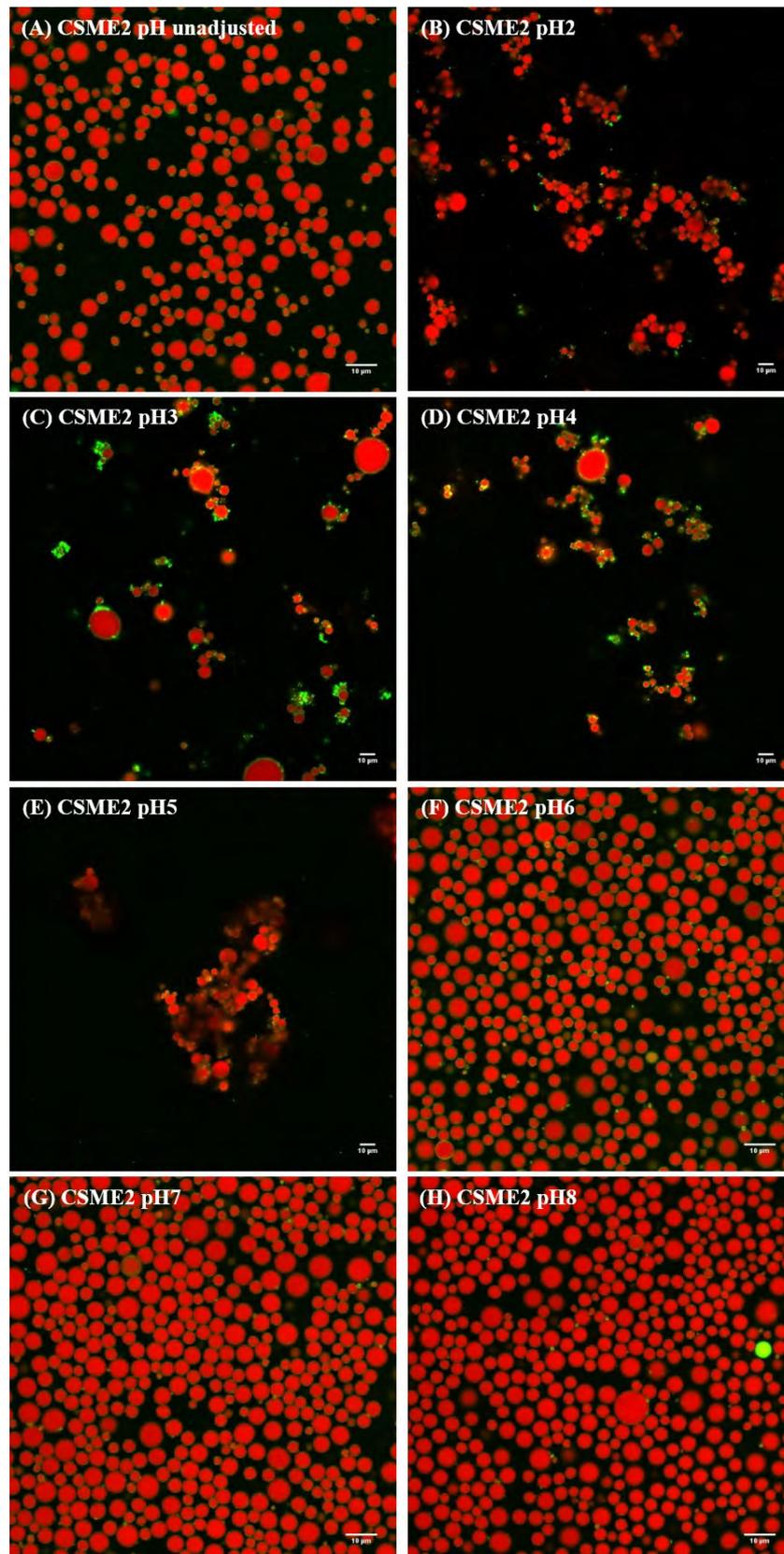


Figure 6.5 CLSM images of coconut skimmed milk extracts formed emulsion (CSME2 at 0.8%, w/w, protein) stained by Nile Red and Fast Green at different pH conditions (pH 2 - 8) (Scale bar = 10 μm)

The CLSM images of COBM1 and COBM2 emulsions (0.4 and 0.8%, w/w, protein) at pH 2 to 8 are shown in Figures 6.6 and 6.7. At all pH values investigated in the study (pH 2 to 8), the droplets of both COBM1 and COBM2 emulsions remained relatively constant size with droplet diameters below 4 and 2 μm , respectively, indicating that the droplets were stable to the changes in pH. There was minimum flocculation in the emulsions at the extremes of pH, in agreement with the particle size data (Figure 6.3). Close to the isoelectric point of the emulsions (pH 4 and 5), the significant flocculation occurred. This indicated that flocculation was predominant in the particle size increases.

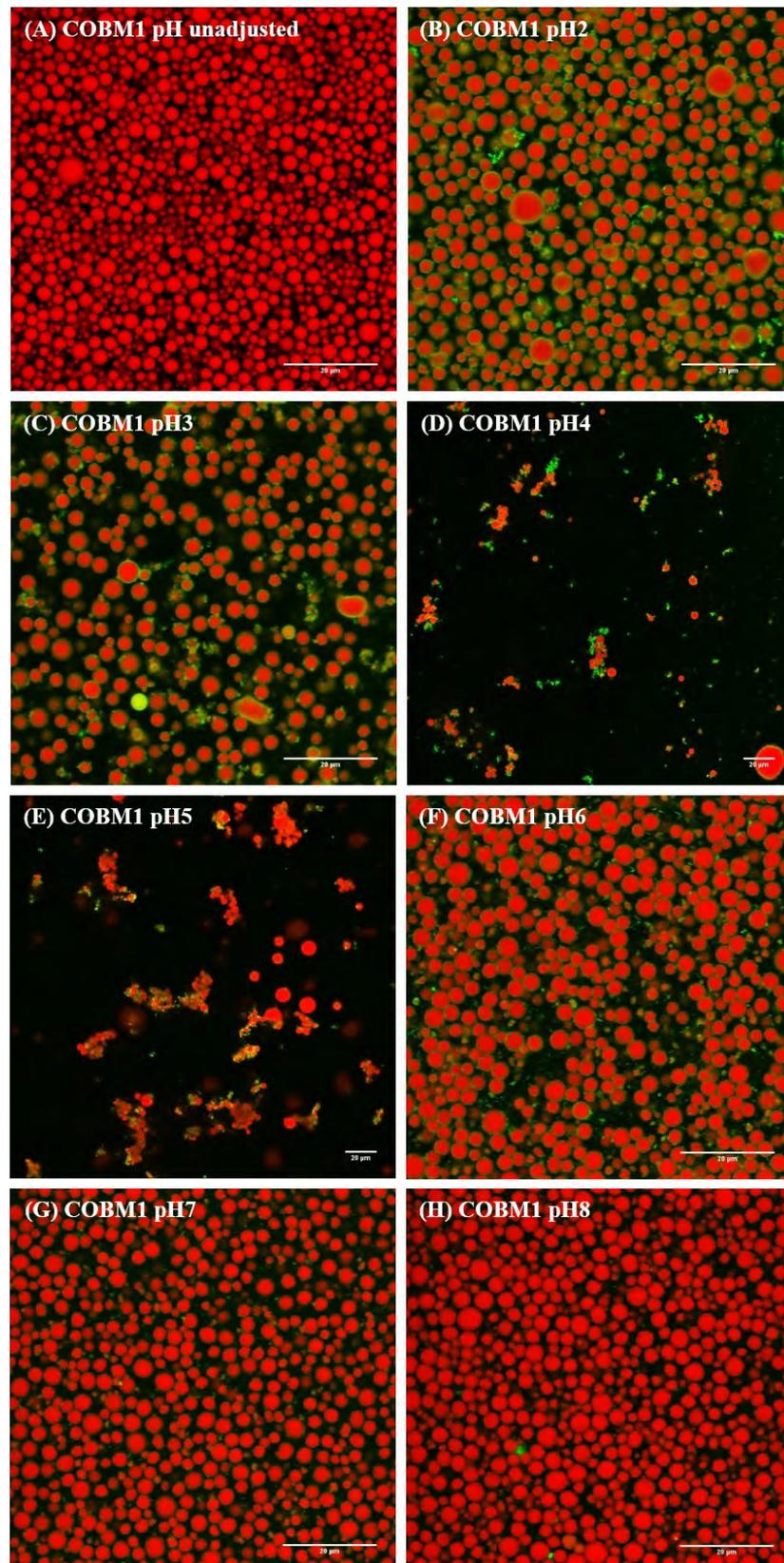


Figure 6.6 CLSM images of coconut oil body membrane extracts formed emulsion (COBM1, 0.4%, w/w, protein) stained by Nile Red and Fast Green at different pH conditions (pH 2 - 8) (Scale bar = 20 μ m)

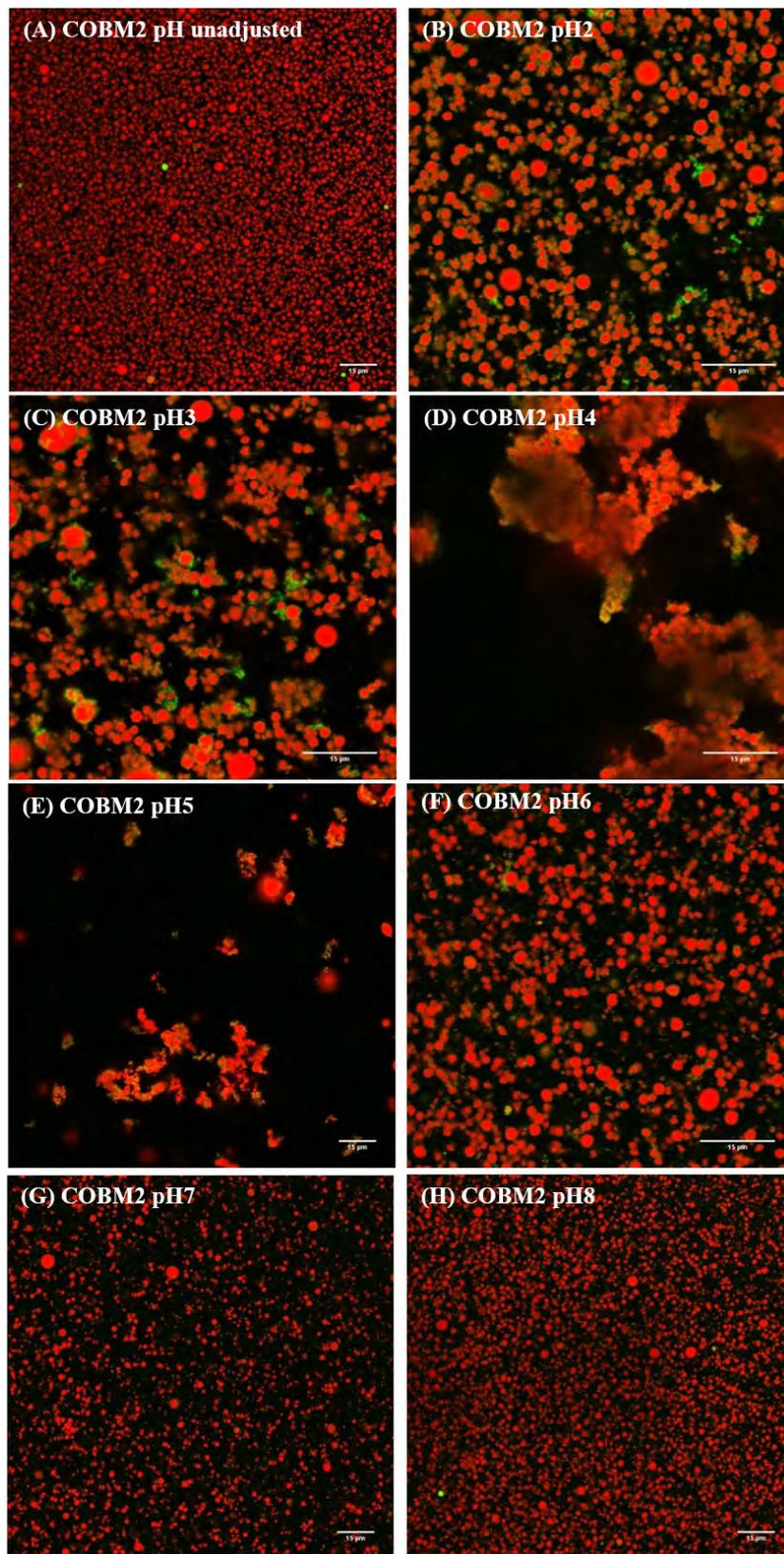


Figure 6.7 CLSM images of coconut oil body membrane extracts formed emulsion (COBM2, 0.8%, w/w, protein) stained by Nile Red and Fast Green at different pH conditions (pH 2 - 8) (Scale bar = 15 μm)

6.2.2 Effect of ionic strength on emulsions

The effect of ionic strength on the properties of CSME emulsions at 0.4 and 0.8%, w/w, protein (CSME1 and CSME2 emulsions, respectively) and COBM emulsions at 0.4 and 0.8%, w/w, protein (COBM1 and COBM2 emulsions, respectively) was investigated by adjusting the ionic strength of CSME and COBM emulsions to different NaCl concentrations (0 to 500 mM). The change in the ζ - potential of CSME2 and COBM2 emulsions at different NaCl concentrations is shown in Figure 6.8.

The CSME2 emulsion had a ζ - potential of -21 mV without the addition of NaCl. As the concentration of added NaCl increased, the ζ -potential of droplets decreased, reaching a value of -7 mV at 100 mM NaCl. A further increase in NaCl concentration from 100 to 500 mM caused only a small decrease in the ζ - potential (a net decrease of -2 mV).

In comparison, the COBM2 emulsion had a higher charge (-35 mV) than that of CSME2 emulsions without the addition of NaCl. At relatively low NaCl concentration (from 0 to 25 mM NaCl), the ζ - potential decreased steeply to around -16 mV, followed by a moderate decrease to about -7 mV with the further increase in NaCl concentration from 25 to 100 mM NaCl. Similar with CSME2 emulsion, a slight decrease in ζ - potential was observed from 100 to 500 mM NaCl. For both CSME and COBM emulsions, NaCl was unable to screen all the charge on the droplets, even at 500 mM NaCl.

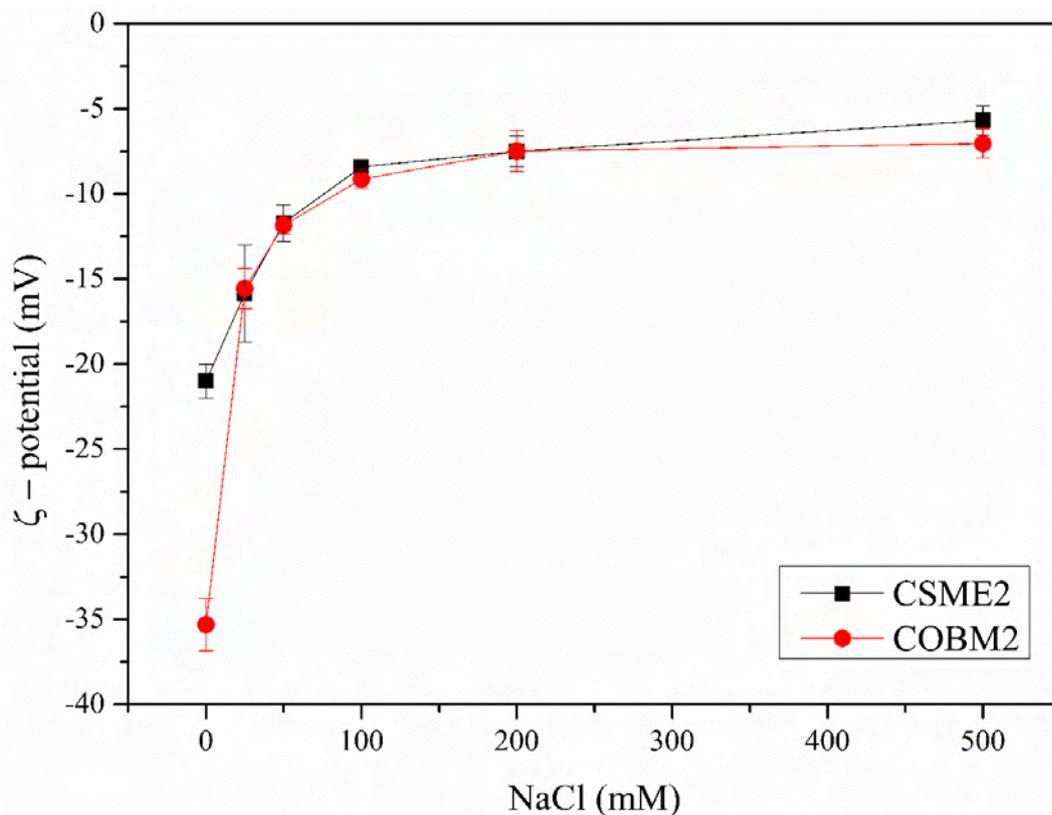


Figure 6.8 ζ - potential of coconut skim milk extracts (CSME2) and coconut oil body membrane (COBM2) emulsions (0.8%, w/w, protein) at different NaCl concentrations (0 to 500 mM)

The effect of different NaCl concentrations (0 to 500 mM) on the particle size ($d_{4,3}$) of CSME1 (0.4%, w/w, protein) and CSME2 (0.8%, w/w, protein) emulsions are shown in Figure 6.9. Increasing the NaCl concentration (0 to 200 mM NaCl) resulted in a moderate increase in the particle size of CSME1 and CSME2 emulsions from approximately 11 and 8.8 μm to 13.5 and 10.5 μm , respectively. CSME1 and CSME2 emulsions dispersed in SDS solution, the particle sizes increased slightly from around 9.2 and 4.6 μm to 10.9 and 5.8 μm , respectively between 0 to 200 mM NaCl. The particle sizes were largely unaffected beyond 200 to 500 mM NaCl. This result suggests that CSME emulsions were slightly flocculated at the low ionic strength and keep stable at high ionic strength.

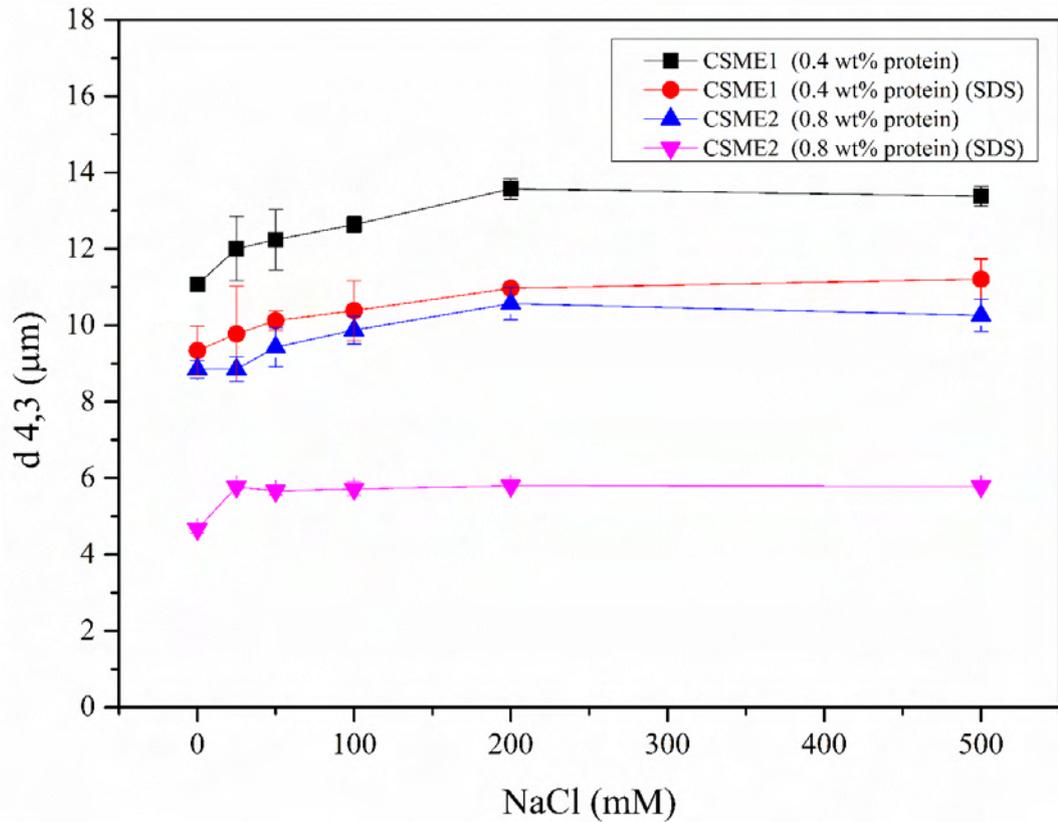


Figure 6.9 Particle size ($d_{4,3}$) of coconut skimmed milk extracts (CSME) formed emulsions at two protein concentrations (0.4 and 0.8%, w/w) at 0 to 500 mM NaCl concentrations and their emulsions in 1% SDS solution

The effect of different NaCl concentrations (0 to 500 mM) on the particle size ($d_{4,3}$) of COBM1 (0.4%, w/w, protein) and COBM2 (0.8%, w/w, protein) emulsions are shown in Figure 6.10. The particle size of COBM1 and COBM2 emulsions (without treated by SDS solution) moderately increased from 2.2 and 1.3 to 4.6 and 2.8 μm , respectively at low NaCl concentrations (from 0 to 50 mM), followed by a dramatic increase to 11.4 and 8.6 μm , respectively, from 50 to 200 mM NaCl. Beyond 200 mM NaCl, the particle size remained constant at approximately 10 μm and 9 μm , respectively. The particle sizes of COBM1 and COBM2 emulsions dispersed in SDS solution were almost the same at approximately 2 and 1 μm , respectively, at all NaCl concentrations (from 0 to 500 mM).

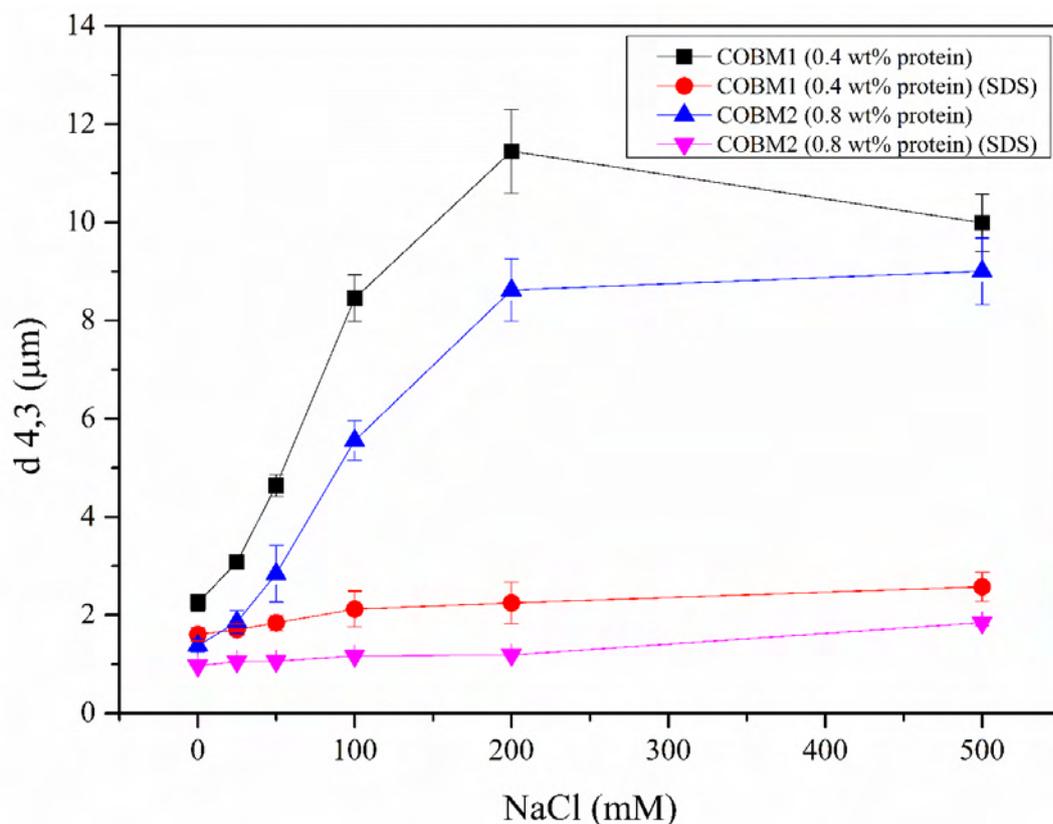


Figure 6.10 Particle size ($d_{4,3}$) of coconut oil body membrane extracts formed emulsion (COBM) at two protein concentrations (0.4 and 0.8%, w/w) at 0 to 500 mM NaCl concentrations and their emulsions in 1% SDS solution

The CLSM images of CSME1 (0.4%, w/w, protein) and CSME2 (0.8%, w/w, protein) emulsions at 0 to 500 mM NaCl concentrations are shown in Figure 6.11 and Figure 6.12. In all NaCl concentration range, the majority droplet size of both CSME1 and CSME2 emulsions remained constant at approximately 8 and 4.5 μm , respectively. According to the particle size data, the flocculation of CSME emulsions was relatively weak, therefore it is not able to see apparent flocculation in CLSM images. With the increase of NaCl concentration, the droplets did not appear to fuse together, which indicate that the CSME emulsions were stable to against NaCl induced coalescence.

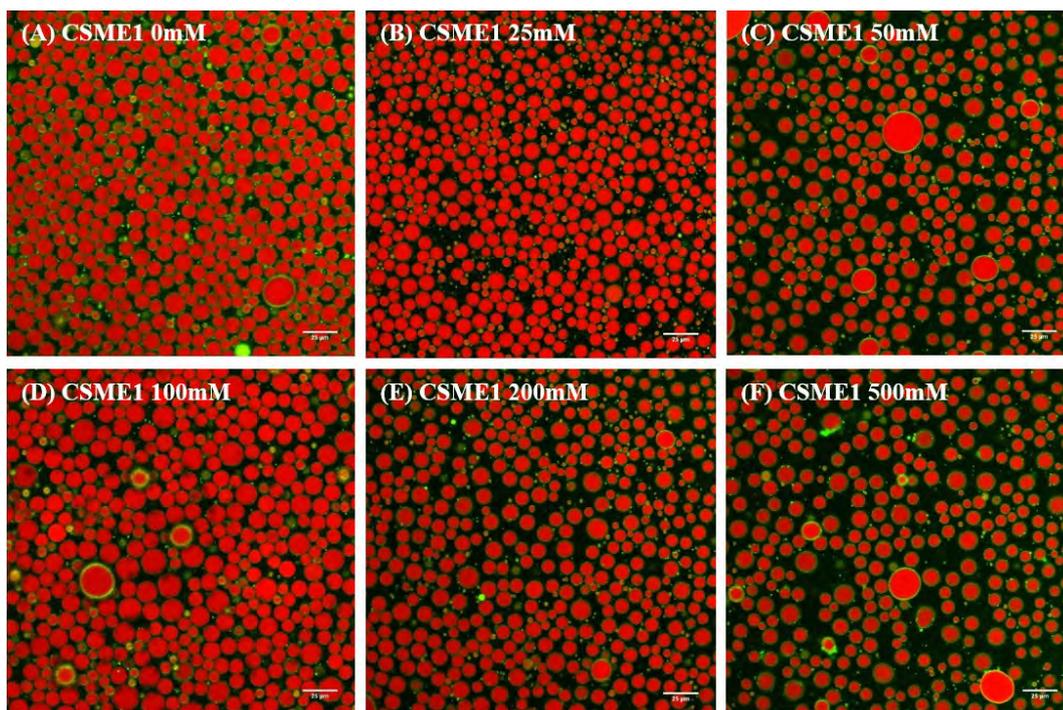


Figure 6.11 CLSM images of coconut skimmed milk extracts formed emulsion (CSME1, 0.4%, w/w, protein) stained by Nile Red and Fast Green at different NaCl conditions (0 to 500 mM) (Scale bar = 25 μ m)

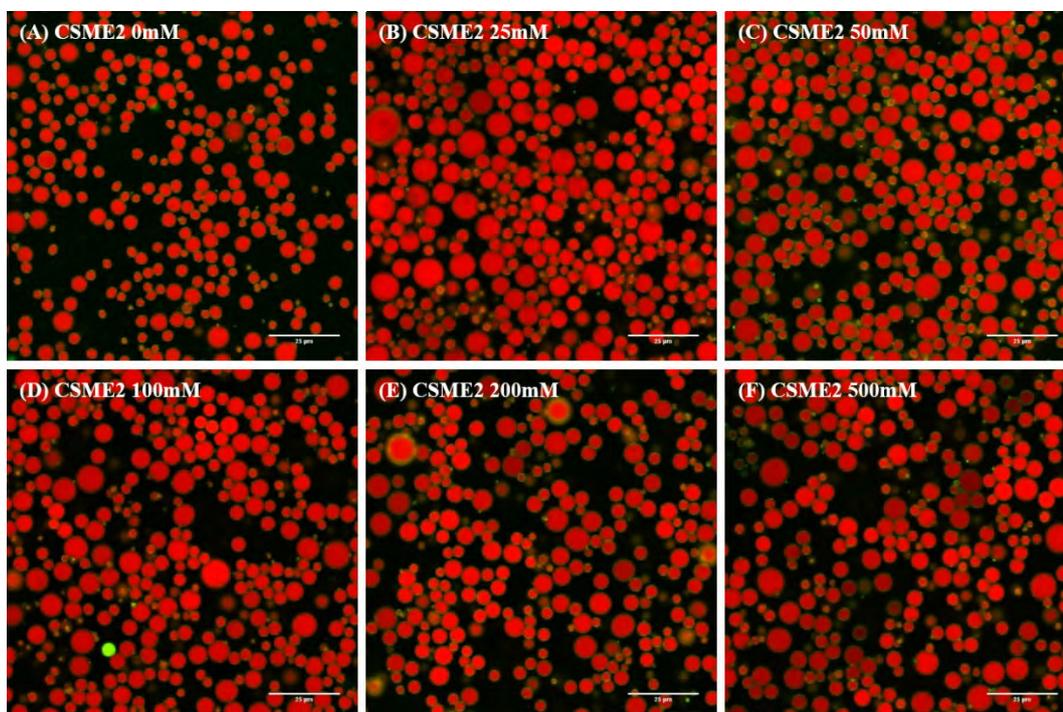


Figure 6.12 CLSM images of coconut skimmed milk extracts formed emulsion (CSME2, 0.8%, w/w, protein) stained by Nile Red and Fast Green at different NaCl conditions (0 to 500 mM) (Scale bar = 25 μ m)

The CLSM images of COBM1 (0.4%, w/w, protein) and COBM2 (0.8%, w/w, protein) emulsions at 0 to 500 mM NaCl concentrations are shown in Figure 6.13 and Figure 6.14. At all NaCl concentrations, the droplet of both COBM1 and COBM2 emulsions remained relatively constant below about 4 μm and 2 μm , respectively. At low NaCl concentrations (0 to 50 mM), the droplets were more or less free of flocculation but appeared to be flocculated at higher NaCl concentrations (50 to 500 mM). This suggested that there was little effect of NaCl concentration on emulsion flocculation at low concentration (0 to 50 mM NaCl), while high NaCl concentrations (50 to 500 mM) resulted in flocculation of droplets.

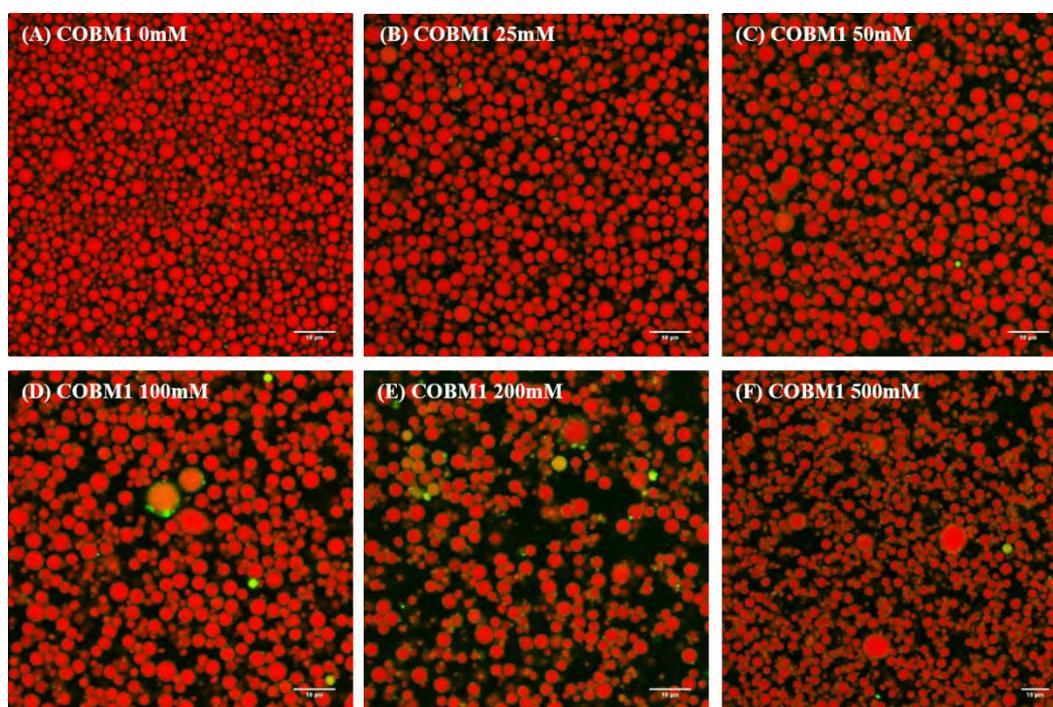


Figure 6.13 CLSM images of coconut oil body membrane extracts formed emulsion (COBM1, 0.4%, w/w, protein) stained by Nile Red and Fast Green at different NaCl conditions (0 – 500 mM) (Scale bar = 10 μm)

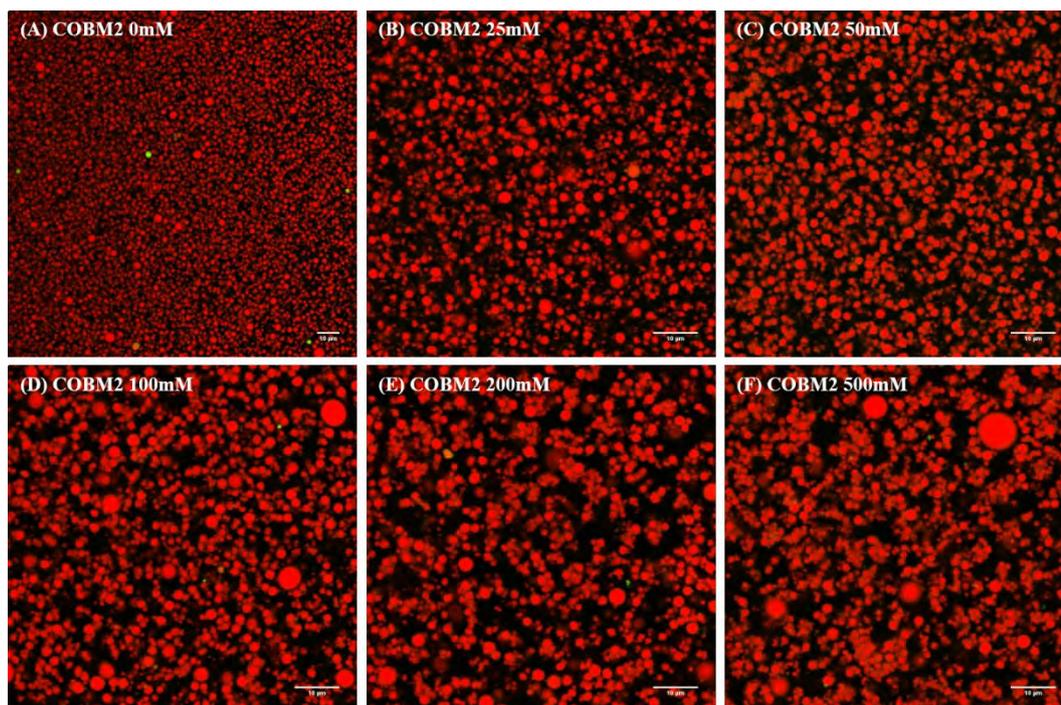


Figure 6.14 CLSM images of coconut oil body membrane extracts formed emulsion (COBM2, 0.8%, w/w, protein) stained by Nile Red and Fast Green at different NaCl conditions (0 – 500 mM) (Scale bar = 10 μ m)

6.3 Discussion

In both CSME and COBM emulsions, the proteins presenting on the surface of emulsion droplets mainly decided the emulsion properties, since protein was the predominant component in the respective extracts. The isoelectric point for CSME and COBM emulsions were between pH 4 and 5, which is in agreement with the results from Chapter 4. Meanwhile, these values were also close to the results from other studies. Onsaard et al. (2005) and Onsaard et al. (2006) found that the isoelectric point of coconut skimmed milk protein concentrate (CSPC) emulsion and coconut cream protein (CCP) emulsion are at about pH 4.5 and pH 4.3, respectively.

Changing pH and ionic strength could affect the ζ - potential of emulsions and the electrostatic repulsion between emulsion droplets. The principle of the changes in ζ - potential induced by pH and NaCl could be explained as follow. At low pH environment (relatively high H^+ concentrations), the protonation of $-NH_2$ happened where the $-NH_3^+$ increased, while the most of $-COOH$ was neutral, which result in more positive ζ - potential. Conversely, at high pH environment, $-NH_3^+$ become neutral $-NH_2$ and the

ionization of the $-\text{COOH}$ result in the rise of $-\text{COO}^-$, which lead to more negative ζ -potential (Chanamai & McClements, 2002). Therefore, if the pH of the emulsion is close to its isoelectric point, the opposite charges tend to be balanced and the electrostatic repulsion will disappear. When increasing the ionic strength, the counter ions (Na^+ in this study) were introduced into emulsion, which could screen the negative charge on the surface of the droplet, resulting in the decrease in droplet charge and low electrostatic repulsion between emulsion droplets (McClements, 2015).

Both CSME and COBM emulsions were relatively stable at high pH environment ($\text{pH} > 6$). This is because the high electrostatic repulsion between emulsion droplets stabilized the droplets against flocculation and coalescence. However, when reduced the pH of CSME and COBM emulsions to their isoelectric point, coalescence happened in CSME emulsions and flocculation happened in COBM emulsions. This could be explained by that electrostatic repulsion provided by surface protein was not enough to overcome the hydrophobic and van der Waals interactions, thus the emulsion droplets were close to each other and flocculation happened (McClements, 2015; Moreau, Kim, Decker, & McClements, 2003). The different types of proteins presenting on the droplet surface and the steric hindrance provided by the surface protein layer of emulsions may decide whether the emulsion will further coalesce. The surface protein layer of CSME emulsions, which are likely to contain a mixture of globulin and albumin proteins (Tangsuphoom & Coupland, 2009), maybe not able to provide enough steric hindrance to protect emulsion from coalescence. However, the predominant proteins in COBE emulsions, which were extracted from coconut cream surface protein, are likely to contain mostly globulins (Tangsuphoom & Coupland, 2009). McClements (2004) suggested that globulins are generally able to form a thicker protein layer at the interface and prevent coalescence. The increase in protein concentration increased the stability of CSME emulsion against coalescence. This may be because higher protein concentration provides more surface protein to stabilize CSME emulsion. The protein concentration did not affect the behaviours of COBM emulsions in different pH, indicating that at least 0.4 % (w/w) protein in COBM emulsion may be sufficient to protect emulsion droplets.

When increasing the NaCl concentration in CSME and COBM emulsions (up to 500 Mm NaCl), both CSME and COBM emulsions (at all given protein concentration) showed flocculation, which could be attributed to the effect of electrostatic screening and enough

steric hindrance provided by surface protein layer mentioned above. Unlike CSME emulsions showed coalescence at low pH, the CSME emulsions were largely free of any coalescence at all given NaCl concentrations. One possible reason may be that the coconut proteins have low protein solubility at low pH conditions (the minimum protein solubility was around 10%, w/w, at pH 3.9) but have high solubility at high ionic strengths (the protein solubility was approximately 90% (w/w), even in 1 M NaCl solution at pH 6 to 8) (Samson et al., 1971). This feature may result in the different stability of the surface protein layer of CSME emulsions under different pH and ionic strength conditions.

6.4 Conclusion

This work showed that coconut oil body membrane (COBM) has better emulsifying properties than coconut skimmed milk extracts (CSME). The COBM emulsions are more stable to changes in the composition of the aqueous phase surrounding the droplets than CSME emulsions. Although, both CSME and COBM emulsions were relatively stable at high pH, COBM emulsions had comparatively better stability at its isoelectric pH. At different NaCl concentrations, the CSME and COBM emulsions did not coalesce, but COBM emulsions had high tendency to flocculate because of the electrostatic screening effect, but this effect was less pronounced in CSME emulsions.

7. Conclusions and avenues for future work

7.1 General discussion

This study compared the emulsifying properties of different biomaterials obtained from coconut milk. The extraction methods standardised in this study could successfully extract the coconut skimmed milk extracts (CSME) and coconut oil body membrane (COBM). In addition, this work addresses the utilization of byproducts obtained during the production of virgin coconut oil.

Both the extracts contained mainly proteins but also phospholipids. This is consistent with another study that obtained both proteins and phospholipids from the surface membrane of plant oil bodies (Tzen & Huang, 1992). The content of proteins in CSME was higher than that in COBM, while the proteins of CSME had lower protein solubility in water than the proteins of COBM. The different types of proteins in CSME and COBM, noted by the SDS-PAGE analysis, were mainly responsible for the differences in their properties, such as solubility, ζ -potentials, protein aggregation behaviour and optical density at different pH. The isoelectric point of COBM solution was higher than that of CSME solution, while the CSME and COBM solutions had similar pattern of pH-induced ζ -potential changes (positively charged at $\text{pH} < \text{pI}$ and negatively charged at $\text{pH} > \text{pI}$). The greater extent of decrease in the interfacial tension between soybean oil and water was achieved by COBM. This suggests that COBM was more surface active than CSME.

Based on the CLSM images of CSME and COBM emulsions and the data from Chapter 4 and 5, it is possible to get detailed information about the structure of CSME and COBM emulsions droplets. Although the phosphorus content in COBM powder was lower, more phospholipids may participate in the stabilization of COBM emulsions. The schematic representation of CSME and COBM emulsions droplets is shown in Figure 7.1.

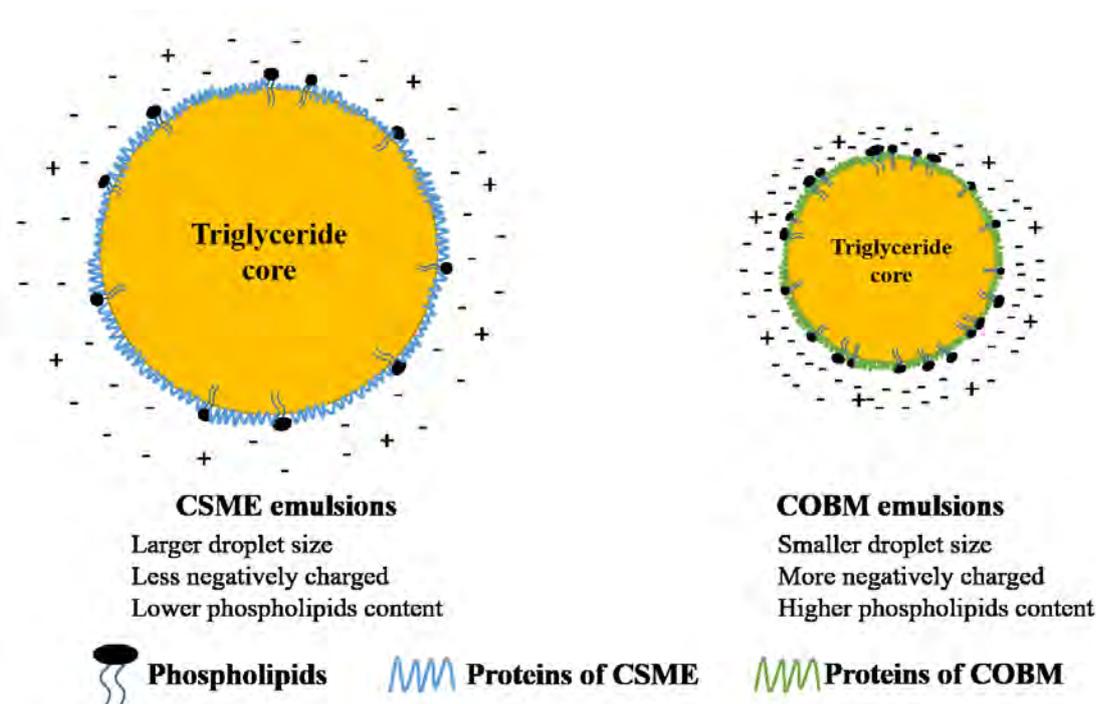


Figure 7.1 The droplet structures of coconut skim milk emulsion (CSME) and coconut oil body membrane (COBM) emulsions.

In Chapter 5, the (20%, w/w) oil emulsions stabilized by CSME or COBM were successfully formed at different protein concentrations (0.2 to 0.8%, w/w). Compared with other studies, 10% (w/w) oil emulsions stabilized by coconut skimmed milk protein concentrate (CSPC) and coconut cream protein (CCP) was formed (Onsaard et al., 2005, 2006). At same protein concentration (0.2%, w/w), compared with CSPC and CCP emulsions, CSME and COBM emulsions achieved smaller particle size, respectively, even at higher oil loading content (20%, w/w).

From the SDS-PAGE analysis, the most prominent band in the surface protein of CSME emulsions was around 50 kDa, while the COBM showed three bands with molecular weights around 55, 37 and 15 kDa. The SDS-PAGE analysis indicates that not all the proteins, which are present in CSME and COBM, participated in the emulsion formation. In addition, the SDS-PAGE of COBM emulsions showed that the interfacial protein composition of COBM emulsions may be similar to natural coconut oil bodies.

The freshly formed CSME or COBM emulsions were at pH 6.4 and 7.8, respectively. At these pH values, the ζ - potential of COBM emulsion (about -35 mV) were higher than that of

CSME emulsion (about -21 mV). In Chapter 6, we note that at a given pH the ζ - potential of COBM emulsion was always higher (more negative or positive) than that of CSME emulsion.

At a given protein concentration, the droplet size of COBM emulsion was always smaller than that of CSME emulsion. This may indicate that COBM had better emulsifying capacity than CSME. The effect of protein concentration on droplet size was more pronounced in CSME emulsions, while COBM emulsions were relatively independent of protein concentrations and COBM can form smaller emulsion droplets at relatively low protein concentration. Thus, to reach the similar particle size, the more an amount of CSME ($\geq 1\%$, w/w, protein) is expected. In addition, the CSME emulsions had higher flocculation degree than COBM emulsions, which may be explained by bridging flocculation induced by insufficient proteins and lower surface charge. COBM emulsions were stable against creaming and remained largely unaffected in particle size even after 30 days at 4°C. These results also suggest that COBM is a better emulsifier in terms of stabilizing emulsions without any environmental change.

The CSME emulsions had a tendency to coalesce at pH < 4. The increase in protein concentration increased the stability of CSME emulsion against coalescence. This may be because, at low protein concentration, the protein adsorbed at droplet surface was insufficient to protect the emulsion. As the protein concentration increased, the amount of protein at the interface increased which contributed to stabilization. In comparison, there was little effect of protein concentration on CSME emulsion stability at NaCl treatment. Both decrease in pH and change in the ionic strengths are expected to screen the charges and are expected to have a similar effect. However, the CSME emulsions remained stable to coalescence even at high ionic strengths, since the ζ - potential on emulsion droplets remained negatively charged, even at the highest concentration of NaCl (500 mM).

The protein concentration did not affect the behaviours of COBM emulsions in different pH and NaCl conditions. Flocculation only happened in COBM emulsions between pH 3 to 6. The droplets of COBM emulsions were flocculated at high ionic strengths but were largely free of coalescence. These results suggest that there was sufficient protein presenting at droplet surface to protect emulsion. Overall, COBM emulsions had better

performance at protecting emulsions from pH change than CSME emulsions, which make COBM as a suitable ingredient for food applications.

Compared with similar studies (Onsaard et al., 2005, 2006), the effect of pH and NaCl concentration on 10% (w/w) oil emulsions stabilized by coconut skimmed milk protein concentrate (CSPC) and coconut cream protein (CCP) (0.2 %, w/w) was investigated. The phenomena of change in particle size of CSPC and CCP emulsions induced by pH and the change in particle size of CCP emulsions induced by NaCl were agreed with our study, while our study further explained whether the coalescence or flocculation dominated the particle size increase. The only difference is that the CSME emulsions (0.4 and 0.8%, w/w protein) were free to coalesce in all NaCl concentrations, while the $d_{4,3}$ values of CSPC emulsion increased with the increase in NaCl concentration. The possible reason may be that there was less protein (0.2 %, w/w) in CSPC emulsion, which was insufficient to protect CSPC emulsion from coalescence.

Compared with other commonly used food emulsifiers, the particle size of 0.7 % (w/w) WPI or sodium caseinate stabilized soybean oil emulsions with higher oil loading content (30 %, w/w) was about 1.1 μm (Ye, Lo, & Singh, 2012), which is similar to the particle size of COBM emulsions (0.8 % protein and 20% oil). It seems that WPI and sodium caseinate may have better emulsifying capacity than COBM and CSME, while it must be noted that the emulsification methods, input energy and environment conditions should also be considered. Thus, the comparison of COBMA and CSME with other emulsifiers is worth to be investigated.

Both pepsin and phospholipase A₂ treatment of CSME and COBM emulsions resulted in droplets coalescence, which suggests the possible droplet interfacial layer structures of CSME and COBM emulsions are similar, that both phospholipids and protein sequences with aromatic and hydrophobic residues present at the interface. The COBM emulsions were more sensitive to the PLA₂ treatment than CSME emulsions, which may suggest that there were more phospholipids presenting on the interfacial layer of COBM emulsions than CSME emulsions. Although the proximate composition of COBM had a lower total phosphorous content than CSME, it is possible that the higher total phosphorous content in CSME was contributed by the residual inorganic phosphorous.

Combining the results in Chapter 5 and 6, the CSME emulsions treated by pepsin had a greater increase in particle size (to 59 μm) than that treated by pH adjustment alone (increased to 22 μm , at pH 2). From SDS PAGE results (Figure 5.11, i, Chapter 5), the predominant band (50 kDa) of the surface proteins of CSME emulsions appeared to be faint upon pepsin treatment and several small bands with molecular weights < 25 kDa appeared concomitantly. From these results, it is possible to conclude that the low pH environment promoted the destabilization of CSME emulsions, while it is not known whether the pH-induced destabilization altered the membrane structure, which in turn facilitated the protein cleaved by pepsin. As for COBM emulsions, the pepsin may be the main factor of the destabilization of emulsions, since there was no effect of low pH environment (pH 2) on particle size.

Both CSME and COBM emulsions remained more or less stable in the presence of trypsin. The stability of COBM emulsion to coalescence is expected, since the oleosin proteins are highly hydrophobic and consist of only few positively charged residues for the action of trypsin (Dave et al., 2019). The stability of COBM emulsion is possible because that positively charged groups were shielded and only few positively charged residues are contained on the surface of CSME emulsion.

From the results in Chapter 5 and 6, it is possible to predict the properties of CSME and COBM emulsions in food formulations and during gastrointestinal digestion. In the formulations of some food, such as sauces and yoghurts where the pH is low, the COBM emulsions may have better stability than the CSME emulsions. To improve the stability of CSME emulsions, some other approaches could be attempted such as complexation with polysaccharides like pectin.

When CSME and COBM are in gastric digestion, both two emulsions will be exposed to the low pH environments. Meanwhile, pepsin will partially hydrolyse the surface proteins of both CSME and COBM emulsions. Some free fat will be released and be digested by gastric lipase. It must be noted that gastric digestion is a dynamic process, with the gastric fluids, hydrochloric acid and pepsin secreted gradually during the digestion and gastric emptying occur simultaneously. It has been shown using Human Gastric Simulator *in vitro* gastric digestion model (Ye, Cui, Dalgleish, & Singh, 2016) and in *in vivo* systems (Mulet-Cabero, Rigby, Brodkorb, & Mackie, 2017), that the pH drop in the stomach

occurs gradually. This implies that at least some of the droplets of CSME and COBM emulsions are emptied out of the stomach before the optimum pH for pepsin activity is reached. This also means that these droplets would be emptied out of the stomach without much proteolytic cleavage by pepsin. When partially digested CSME and COBM emulsions pass to intestinal digestion, it is not clear that if trypsin is not able to act on surface protein immediately. However, the droplets may be acted upon by the other enzymes of pancreatic juice (chymotrypsin and pancreatic lipase). Meanwhile, phospholipase can digest phospholipids and destabilize emulsions. Trypsin may access to its target protein since the emulsion structure is changed due to the digestion in the stomach and small intestinal.

7.2 Avenues for future work

It should be noticed that this study is the first step of investigating that if the CSME and COBM could be used as emulsifiers in the food area and give us a general idea of the properties and difference of emulsions formed by these two extract materials. There are some limitations in this study and more works are recommend in the future.

1. Quantitation of phospholipids in CSME and COBM

The confocal laser scanning microscopy use for the detection of phospholipids is only a qualitative method. While the measurement of the total phosphorus content for the estimation of phospholipids include both organic phosphorus and residual inorganic phosphorus from the extraction processing. More sophisticated methods such as HPLC and ³¹P NMR could be used for quantitation and for identification of different types of phospholipids.

2. Understanding the performance of CSME and COBM emulsions in the gastrointestinal tract

As discussed above, the enzymatic studies provide insights into the behaviour of CSME and COBM emulsions during gastrointestinal (GI) digestion. The presence of a gastric lipase, bile salts and pancreatic lipase may alter the kinetics of free fatty acid release. More detailed studies that include sequential *in vitro* gastric and intestinal digestions may provide details about the digestion of these emulsions.

3. Bioactive components encapsulation and their effect on bioavailability

Emulsions stabilized by oil body membrane material from sesame seeds have been known to increase the bioavailability of curcumin (Chang et al., 2013). Since the emulsions were successfully stabilized by CSME and COBM, these two materials may help to encapsulate bioactive components in emulsions to increase the health value in food. Thus, the studies of encapsulation efficiency of different bioactive components and their stability during storage are worth to be investigated.

4. Comparison studies to test the stability of bioactive ingredient in nano-emulsions stabilized by COBM and CSME

The stabilization of the bioactive ingredients encapsulated by CSME and COBM is another important character. The comparison studies could be carried out to test the stability of the bioactive ingredients under storage conditions.

5. Heat stability of emulsions

The proteins in COBM and CSME emulsions may be denaturated by thermal processing, which may affect the properties of emulsions. Therefore, it is meaningful to test the heat stability of COBM and CSME emulsions.

6. Exploring foam stabilization properties of COBM and CSME

Foams happened in many food applications, thus the effect of composition difference of COBM and CSME on the foam properties and foam stability are worth to be investigated.

7. Incorporation of emulsions in gels for texturing

Another possible application in food is the incorporation of CSME and OBME in gels. It would be interesting to investigate the effect of CSME and OBME on the texture and functionality on the gels.

8. Interaction of CSME and OBME emulsions with polysaccharides

The droplets of CSME emulsions are expected to be prone to destabilization at low pH conditions, which may make them susceptible destabilization in the stomach. It is not known if the stability of CSME emulsions at low pH could be improved by complexation with polysaccharides like pectin. The interactions of CSME and COBM emulsions with

polysaccharides provide different functionalities and digestion behaviours, which is an interesting area for future research.

9. Commercial application of COBM and CSME

The COBM and CSME were both extracted in a pilot plant, indicating these extraction methods could be used at commercial scale. It is interesting to look at the chance for applying them in commercial food formulations and testing its stability during the operations of food processing e.g. UHT treatment, fermentation.

10. Extraction oil body membrane extracts from other plant food material

A pilot-plant scale method of extraction of the coconut oil body membrane was standardised in this study. Thus, this extraction method may work on other plant food material, such as avocado and almond, to develop some other novel food materials. Since the composition of oil body membrane materials varies from species to species, it is possible that the resulting membrane extracts have different properties. It would be interesting to test this hypothesis and determine the effect on the emulsifying properties.

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