In Vitro Gastrointestinal Digestion of Oil-in-Water Emulsions

A thesis presented in partial fulfilment of the requirements for the degree of
Master of Technology in Food Technology
at Massey University, Auckland
New Zealand

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2012

Abstract

Oil-in-water (O/W) emulsions are widely used as a dispersion system for oil or fat or as a delivery system for lipophilic bioactive compounds in aqueous food products. There is a growing interest among food scientists in understanding the digestion behaviour of O/W emulsions when they are ingested and pass through the gastrointestinal (GI) tract. In recent years, a number of researches have been carried out to investigate the lipid digestion of emulsions using *in-vitro* models such as simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) that mimic the biological conditions of human bodies because of the complexity of *in vivo* study. However, most studies have been conducted to study the effect of gastric or intestinal digestion using SGF or SIF, and the studies on the effect of sequential digestion of emulsions first in SGF and then in SIF have been very limited. The objective of this study was therefore to investigate the effect of *in vitro* digestion of emulsions sequentially in SGF and SIF on the physicochemical properties and lipolysis of emulsions. In this study, sodium caseinate, whey protein isolate (WPI) and Tween 20 were used as emulsifiers to prepare O/W emulsions (20% soy oil and 1% emulsifier). The mean particle size and particle size distribution, zeta potential and microstructure of freshly prepared emulsions were initially measured, and the changes in the physicochemical properties of emulsions occurring during digestion in SGF or SIF and sequentially in SGF and SIF were analysed. The hydrolysis of fatty acids from emulsified lipid core was also determined during digestion in SIF after gastric digestion. In acidic simulated gastric conditions (pH 1.6 and 3.2 mg/mL pepsin), sodium caseinate-stabilized emulsions showed extensive flocculation with some coalescence, resulting in change in the size and microstructure of the emulsions. In contrast, the emulsions stabilized with WPI or Tween 20 showed no pronounced changes over time during 2 hrs of gastric digestion. In simulated intestinal conditions (pH 7.5, bile salts and pancreatin), a massive coalescence by pancreatic lipase took place in both sodium caseinate and WPI-stabilized emulsions, leading to a pronounced increase and change in the droplet size and microstructures, whereas Tween 20-stabilized emulsions were relatively stable with much less droplet coalescence and size increase. After sequential digestion in SGF and SIF, protein-stabilized O/W emulsions showed more extensive aggregation and coalescence of droplets in comparison with their digestion in SIF only without gastric digestion, whereas Tween 20-stabilized emulsions were relatively stable with only some extent of coalescence after 2 hrs of its

sequential digestion in SIF after SGF. The amounts of free fatty acids released in SIF after gastric digestion were similar between three types of emulsions and were not affected significantly by the gastric digestion prior to the intestinal digestion. The overall results indicated that the digestion behaviour of emulsions was affected by types of emulsifiers, and that the sequential digestion of emulsions in SGF and SIF resulted in more pronounced changes in the emulsion particle size and microstructure compared to the digestion in SGF or SIF. However, the rate of lipid digestion was not affected by the sequential digestion. The results of this study provide a significant insight into the effect of sequential gastric and intestinal digestion on the size and properties of emulsion systems and its effect being different depending on type of emulsifiers used to stabilise oil droplets.

Acknowledgements

I am sincerely thankful to my chief supervisor Dr. Sung Je Lee for giving me the opportunity to study for my masters in Food Technology at Massey University. The research present in this thesis would not have been possible without his patient guidance, consistent encouragement, and great help. I am very grateful for his scientific comments and academic advice on my research and writing up this thesis. It was a great experience to work under his supervision.

I am sincerely thankful to my co-supervisor Dr. Ai Qian Ye (Riddet Institute) for his support and guidance during my research at Riddet Institute. His continued encouragement and help and his intelligent ideas and scientific knowledge in emulsions have been a great help in completing my research project.

I am truly thankful to Professor Harjinder Singh for giving me the chance to conduct my research project at Riddet Institute with a financial support.

I would like to thank Riddet Institute laboratory managers Ms. Janiene Gilliland and Mr Chris Hall for their support (trainings, laboratory induction) and help in laboratory work. I would like to offer my special thanks to Jack Cui for his guidance on my overall practical work and his consistent encouragement during my study. I would also like to thank Mr Garry Radford (IFNHH) for his guidance on using homogenizer, Dr Jianyu Chen for her patient guidance on using Laser Scanning Confocal Microscopy (LSCM) and my officemates, Mr Mallesh Peram and Mr Anant Dave, for their great help in running SDS-PAGE gels.

My special thanks also go to my flatmates, Weifong Pok, Karren Khaw and Xianhui Geng, for their encouragement, accompany and support during my study. I would also like to offer my great thanks to all my officemates, Marllesh Peram, Anant Dave, Sylvia Chung, and Davastotra Poddar, for their academic advices and support.

Last but not the least, I would like to thank my beloved parents and sister for their unconditional support and continuous encouragement whenever in need.

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Chapter 1. Introduction

There is a growing interest in understanding how lipid digestion can be controlled under physiological conditions which might be able to help with calories intake regulation and satiety responses (McClements et al., 2009a, Singh et al., 2009, Golding and Wooster, 2010). Lipids found in many foods exist in the form of emulsified oil droplets, including salad dressings, mayonnaise, spreads, ice cream and soups (McClements, 2005, Singh et al., 2009). Food emulsions are stabilized by emulsifiers, known as surface-active substances that are capable of adsorbing at the oil-water interface and preventing the occurrence of aggregation of oil droplets (McClements, 2005). The most commonly used food emulsifiers are protein or polysaccharide-based emulsifiers and small molecule surfactants (Singh et al., 2009).

The stability of emulsions stabilized by proteins or surfactants during processing and their interactions with biopolymers (carrageenans, dextran, guar gum, etc) were largely reported (Dalgleish, 2003, Dickinson, 1998, Dickinson et al., 1998, Srinivasan et al., 2002, McClements, 2004, Bibette and Leal-Calderon, 1996, Dickinson, 1999, Dickinson, 1997, Dalgleish, 1997, Dickinson et al., 1999, Dimitrova and Leal-Calderon, 1999). Recently, the *in vitro* digestion of emulsions has raised a great interest among scientists. Many studies have been carried out to establish the mechanisms of emulsified lipid digestion and absorption in the gastrointestinal tract (Hu et al., 2011, van Aken et al., 2011, McClements et al., 2009b, Sarkar et al., 2009a, Sarkar et al., 2009b, Sarkar et al., 2010b, Sarkar et al., 2010a, Singh et al., 2009). However, most of these studies have focused on the *in vitro* gastric digestion or intestinal digestion of emulsions (Sarkar et al., 2009b, Sarkar et al., 2010b, Sarkar et al., 2009c, Mun et al., 2007, Malaki Nik et al., 2010b, van Aken et al., 2011). There is limited information about the lipid digestion of emulsions during sequential gastric and intestinal digestion. Therefore, the objective of this study was to examine the lipid digestion and physicochemical changes of emulsions occurring during digestion sequentially in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Oil-in-water (O/W) emulsions stabilized by three different types of emulsifiers, which are proteins (sodium caseinate and whey protein isolate) and non-ionic surfactant (Tween 20), were prepared and analysed for the determination of changes in the size, zeta potential, microstructure and interface composition of oil

droplets during sequential digestion. The amount of free fatty acids hydrolyzed from emulsified lipids was also measured.

Chapter 2. Literature Review

2.1 Introduction

An emulsion is defined as a colloidal suspension consisting of two immiscible liquids in which one liquid (dispersed phase) is dispersed as small droplets in another liquid (continuous phase). There are two common types of emulsions found in foods, such as an oil-in-water (O/W) emulsion and a water-in-oil (W/O) emulsion. In O/W emulsions, oil is dispersed as droplets in the continuous aqueous phase of water, whereas W/O emulsions have water droplets dispersed in the oil phase (Dickinson, 2010b). The size of dispersed droplets can be 0.1-100 μm in diameter (Singh et al., 2009). Emulsions are nowadays widely used in the food products, including milk, dairy cream, salad dressings, mayonnaise, spreads, ice cream and soups (McClements, 2005, Singh et al., 2009).

The droplets of O/W emulsions dispersed in the continuous phase are stabilized by amphiphilic surface-active compounds (emulsifiers) with both hydrophilic and hydrophobic groups. Emulsifiers have the ability to adsorb onto an oil-water interface, reduce the interfacial tension between the two phases, and prevent the occurrence of aggregation of emulsion droplets (McClements, 2005). The commonly used food emulsifiers are some protein and polysaccharide emulsifiers and small molecule surfactants (Singh et al., 2009). Effective emulsifiers are generally capable of rapidly adsorbing onto the surface of emulsion droplets formed by homogenization during the emulsification of oil into the aqueous phase containing emulsifiers and then forming an interfacial layer that prevents the oil droplets from coming into close proximity with each other. The droplet aggregation and coalescence are normally prevented by two different mechanisms, such as electrostatic and steric repulsion forces. The viscoelastic properties of emulsion droplet layers at oil-water interface besides their chemical properties, and the density difference between oil and water phases have also significant influence on the stability of emulsion droplets to aggregation and coalescence (Dickinson, 2010b, Dalgleish, 2004, McClements, 2005). It is therefore important to understand the physicochemical properties of emulsifiers surrounding oil droplets and their changes upon exposure to various environmental conditions.

The interactions between emulsion droplets in response to different environmental conditions (such as mouth, stomach, intestine) was reported to be determined by the structure, thickness, composition and charge of interfacial layers of emulsions (Singh et al., 2009). An emulsion passes through the mouth into the stomach and then the intestine when ingested (Singh et al., 2009). It undergoes a drastic pH change from pH 1~3 up to 7 and mechanical agitation due to peristaltic movement in the stomach. Moreover, emulsions are mixed with gastric fluid exposing to different enzymes (pepsin, gastric lipase) and electrolytes (Na^+, Cl^-, Ca^{2+}, etc) as they enter the stomach (Dickinson, 2010b, Singh et al., 2009, Beysseriat et al., 2006). When a partially digested emulsion enters the small intestine from the stomach, it undergoes a wide range of physicochemical conditions, including mixing with various enzymes, such as pancreatic lipases, and surface active agents, such as bile salts. Moreover, it goes through a substantial change in pH (from 1.5~3.0 in the stomach to ~6.0-7.5 in the intestine) and a variation in ionic strength as the presence of different electrolytes (Singh et al., 2009, McClements et al., 2008). Therefore, the changes occurred in the interfacial layer of the emulsion droplets could be extremely complicated and largely dependent on the concentrations and surface activities of the intestinal components at any given period of time. Model systems are thus used to understand such complex conditions as they allow separate investigations of emulsion digestion behaviors and interactions in the context of individual physiological components.

A number of recent studies have reported the physico-chemical changes of emulsions using either *in vitro* gastric digestion or *in vitro* intestinal digestion models (Sarkar et al., 2009c, Sarkar et al., 2009b, Duffy et al., 2009, Hur et al., 2009, Golding and Wooster, 2010, Sarkar et al., 2010b, van Aken et al., 2011, Malaki Nik et al., 2011, Hur et al., 2011a, Hur et al., 2011b, Mun et al., 2007). However, there is limited information in *in vitro* sequential digestion in both SGF and SIF. The objective of this thesis is to understand the physicochemical changes of O/W emulsions and their lipid digestion during SGF, SIF and sequential digestion in both SGF and SIF. The oil-in-water emulsions (20% oil) coated with three different types of emulsifiers (sodium caseinate, Tween 20 and WPI) were prepared.

This chapter covers a wide range of knowledge on O/W emulsions, emulsion formation, emulsifiers, emulsion stability and factors affecting its stability such as pH, ionic

strength, enzymes etc. Moreover, it also includes *in vitro* gastric digestion and *in vitro* intestinal digestion of O/W emulsions based on current studies and reports.

2.2 Emulsion formation

Emulsion formation involves a single step or a series of consecutive steps, depending on the nature of starting materials employed, size and properties of emulsion droplets to achieve, and methods used to prepare it. The process of converting two immiscible liquids of oil and water into an O/W emulsion is referred to as homogenization that involves a high mechanical force to breakdown bulk oil into small oil droplets. . This can be achieved using a range of devices, such as high shear mixer, blender, ultrasonicator and high pressure value homogeniser. High-pressure valve homogenizer is the most commonly used in the food industry as it is more effective at reducing the size of droplets.

The basic principle of homogenizer is to force a mixture of oil and aqueous phase through a narrow slit with high pressure applied, resulting in cavity, intense laminar shear flow and turbulence that cause the breakdown of oil into small droplets. The small oil droplets formed in the homogenizer are then dispersed in the aqueous phase containing emulsifiers. The emulsifiers adsorb onto oil droplet and form a protecting membrane, thus preventing aggregation and coalescence of droplets when they come into close contact (McClements, 1999, McClements, 2005).

2.3 Emulsion stability

Emulsion stability is the term describing how an emulsion remains its consistency without undergoing changes in the physicochemical properties after the emulsion preparation, during the subsequent storage or upon exposure to various environmental conditions. This means that the more stable the emulsion, the emulsion is more resistant to changes in its properties over time and the more slowly its properties change (McClements, 2005). Emulsion instability can be divided into two types, physical and chemical destabilization. Physical instability includes creaming, flocculation, coalescence and phase inversion, which leads to changes in the structure, spatial distribution and properties of droplets in the emulsion system whereas chemical

instability includes generation of new molecules (e.g. oxidative products) via chemical reactions and degradation (McClements, 2005).

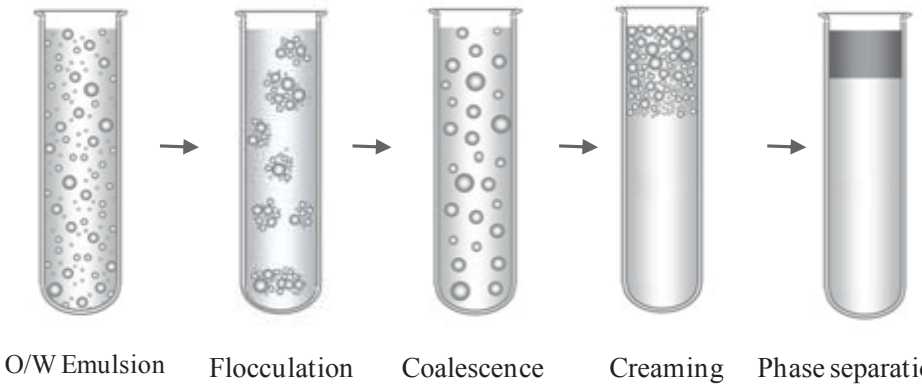


Figure 2.1 Various types of physical destabilization of O/W emulsions.

Emulsion stability is often explained by thermodynamic versus kinetic stability. Thermodynamics indicate whether a given process will occur or not whereas kinetics demonstrates the rate of the process that will proceed if it does occur, and it determines the stability period of emulsions. An emulsion is thermodynamically unstable due to an increase in the interfacial area between oil and water phase (McClements, 2005, McClements, 2000). The change in interfacial free energy between the initial state (separated phase before emulsification) and final state (homogenized state after emulsification) is equal to the increase in contact area between the oil and water phase and normally expressed with mathematical equations given below by comparing free energy between the initial (G^i) and final emulsion (G^f):

In the initial state, $G^i = G_O^i + G_W^i + G_I^i - \mathrm{T}S_{config}^i$ and after emulsification, it is given by $G^f = G_O^f + G_W^f + G_I^f - \mathrm{T}S_{config}^f$

where, G_O, G_W, and G_I are free energies of the oil phase, water phase and the oil-water interface, respectively, T is the absolute temperature, and S is the configurational entropy of the droplets in the system. The superscripts i and f refer to the initial and final states of the system, respectively. Free energies of the bulk oil and water phases always remain constant before and after homogenization: $G_O^i = G_O^f$ and $G_W^i = G_W^f$, as a result, the difference in free energy between the initial and final state can be described as:

$$\Delta G_{formation} = G^f - G^i = G_I^f - G_I^i - (TS_{config}^f - TS_{config}^i) = \Delta G_I - T\Delta S_{config}$$

ΔG_I is also defined as γΔA, whereas A is change in interfacial energy and γ is interfacial tension. It is always a positive term due to the interfacial area that always increases after homogenization. The configurational entropy term ($- T\Delta S_{config}$) is always negative, as the possible arrangements after emulsification are much greater than that of the initial state. As compared to the γΔA, the entropy term is almost 107 times negligible and hence ($- T\Delta S_{config}$) can be neglected. Therefore, the free energy of emulsification can be expressed as: $\Delta G_{formation} = \gamma\Delta A$ (Mahungu and Arts, 2002).

Most food emulsions are thermodynamically unstable systems and will eventually break down after a long-term storage. This mainly will result in different types of physical changes, of which flocculation, coalescence and creaming are the main mechanisms.

Creaming refers to the gravitational separation of emulsified droplets to form a densely packed phase at the top of emulsions (Figure 2.1). The rate of creaming can be predicated by Stoke's equation as in the following:

$$V = \frac{2(\rho 1 - \rho 2) g r^2}{9\eta}$$

where V is the creaming velocity, g is the acceleration due to gravity, r is the radius of the particle, ρ_1 and ρ_2 are densities of continuous phase and dispersed phase, and η is the dynamic viscosity of continuous phase (McClements, 2005). The V determines the kinetic stability of an emulsion, and it can be increased by lowering the radius of droplets, decrease the difference of density between two phases, and increase the viscosity of continuous phase.

However, Stoke's law does not apply in some flocculated emulsion systems in which the network structure is formed or when the surfactant layer on the oil droplets significantly increases the density of dispersed phase (Syrbe et al., 1998).

Flocculation is a process by which two or more droplets come together to form aggregates via weak attractive forces but the droplets retain their individual integrity (Dickinson, 1987, McClements, 2005). Although this process generally modifies the physical properties of emulsion, the droplet size distribution remains unaltered and the

flocs can be readily dispersed due to their weak interactions (Dickinson, 1987, Tcholakova et al., 2006). There are two types of flocculation, which are depletion flocculation and bridging flocculation (McClements, 2005) which is discussed further below.

2.3.1 Depletion flocculation

The presence of non-adsorbing biopolymers and surfactants in the continuous phase of emulsions causes an osmotic effect between droplets, resulting in an increase in the attractive force. The attractive force increases as the concentration of non-adsorbing biopolymers increases, and finally overcomes the repulsive force between droplets, causing the droplets to flocculate. This is called depletion flocculation (McClements, 2004, McClements, 1999, McClements, 2005), which could lead to emulsion instability.

The strongest attraction between the droplets occurs when they come into contact, leading to flocculation and loss of emulsion stability. This attraction energy equation (McClements, 2005, McClements, 2000) is expressed as droplet-droplet interaction potential as following:

$$Wdep\,(0) = -\frac{3kT}{2}\frac{cRv}{\rho}\left(1+\frac{1}{2}\frac{cRv}{\rho}\right)\left(\frac{rd}{rg}+\frac{2}{3}\right)$$

$$Rv = \frac{4\pi\gamma_g^3\,\rho N_A}{3M}$$

where c is biopolymer concentration (kg/m^3), *rd* and *rg* are the radius of the emulsion droplet and the radius of gyration of the biopolymer, respectively, ρ is the density of biopolymer, N_A is the Avogadro number and M is the molecular weight of biopolymer molecule (kg/mol), kT is the kinetic temperature, and Rv is the "effective" volume ratio of polysaccharides (i.e. "effective" volume of polysaccharides in the solutions divided by the actual volume of polysaccharide chain).

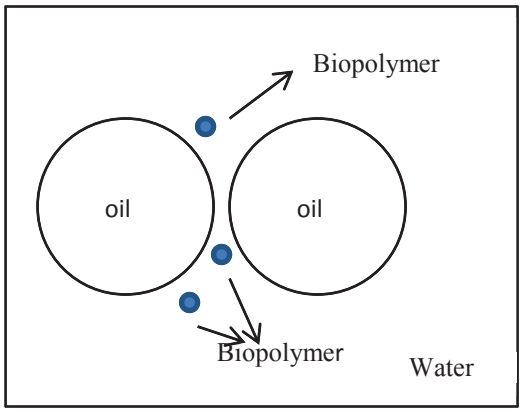


Figure 2.2 Depletion flocculation in an O/W emulsion. Particles approach due to an osmotic pressure gradient pushing out the unadsorbed biopolymer.

With the addition of sufficient high enough concentrations, many types of biopolymers and surfactants are able to cause depletion flocculation, including surfactants (Tween 20, sodium dodecyl sulphate (SDS)), polysaccharides (xanthan gum, gum arabic, pectin, dextrin) and proteins (whey protein isolate and caseinate) (McClements, 2005). Critical flocculation concentration (CFC) refers to the lowest concentration required to cause flocculation, and it decreases as the emulsion droplet size increases and the effective volume fraction of the biopolymers or surfactants increases. The flocculation rate initially increases as the concentration of non-adsorbing biopolymers or surfactants increases due to droplet attraction enhancement. However, once the concentration is high to some extent, the flocculation rate will decrease as the continuous phase is too viscous to allow movement of droplets (McClements, 2004, McClements, 2005). It has been reported that sufficiently high concentrations of non-adsorbed caseinate can also promote emulsion instability through a depletion flocculation mechanism (McClements, 2005).

2.3.2 Bridging flocculation

Many different types of biopolymers, such as xanthan gum, guar gam, ι-carrageenan, dextrin and pectin, have been shown to promote flocculation by charge neutralization and bridging effects between two or more droplets. Electrically charged biopolymers adsorb onto the surfaces of oppositely charged emulsion droplets by electrostatic reactions (Dickinson et al., 1989, McClements, 2005, McClements, 2004, McClements, 1999). Biopolymers may adsorb either directly to the bare surfaces of the droplets or to

the adsorbed emulsifier molecules that form the interfacial membrane (McClements, 2004). There must be a strong attractive force between segments of the biopolymer and the droplet surface for the binding of biopolymers to the droplets. The most common types of reactions are hydrophobic and electrostatic forces (McClements, 1999). A number of researches have been carried out to find out the factors affecting the occurrence of bridging flocculation (Surh et al., 2006, Dickinson and Galazka, 1991, McClements, 2005, McClements, 1999, Dickinson et al., 1989, McClements, 2004).

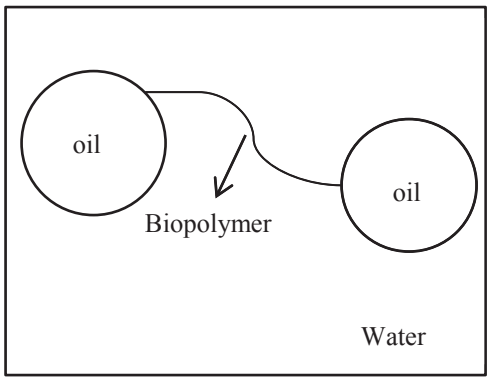


Figure 2.3 Bridging flocculation in an oil-in-water emulsion.

Bridging flocculation can occur when a low concentration of a high molecular weight biopolymer (e.g. polysaccharides) is present in a protein stabilized emulsion and the biopolymer adsorbs onto two or more emulsion droplets either hydrophobic or electrostatic attractive interactions (Dickinson et al., 1989, McClements, 2005, McClements, 2004, Dickinson and Galazka, 1991). When a protein-polysaccharide mixture is present to stabilize an emulsion, a low concentration of polysaccharides present is found to promote bridging flocculation. This was attributed to the formation of polymeric linkages by polysaccharides between protein-adsorbed emulsion droplets (Dickinson et al., 1989, McClements, 2005). An example of this phenomenon is that emulsions stabilized by β-lg undergo bridging flocculation by charged polysaccharides at pH values where the polysaccharide and protein have opposite charges (McClements, 2004). This implies that bridging flocculation could occur when a biopolymer is charged oppositely in the continuous phase to the droplets and its concentration is low.

However, if a high concentration of polysaccharide is present in the continuous phase, bridging flocculation will not likely to occur because the amount of biopolymer is

sufficient to completely cover the droplet surfaces (Dickinson and Galazka, 1991). However, as mentioned above, depletion flocculation may occur if the free biopolymer concentration is too high (McClements, 2005, Dickinson and Galazka, 1991).

Other factors, such as low pH and addition of biopolymer, are found to promote bridging flocculation by charge neutralization when oppositely charged biopolymer is added into emulsions. The influence of pH and pectin type on stability of sodium caseinate-stabilized emulsions was investigated by Surh et al. (2006). It has been reported that at pH values below the pI of the adsorbed casein, negatively charged pectin molecules adsorb to the surface of positively charged caseinate-coated droplets leading to charge neutralization and promoting bridging flocculation. This results in the formation of a strong network of aggregated emulsion droplets and the increase of apparent viscosity and shear thinning of the emulsions (Surh et al., 2006).

2.3.3 Coalescence

Coalescence refers to a process of merging two or more liquid droplets together to form one larger droplet. Coalescence could lead to droplet creaming or sedimentation more quickly due to the increase in droplet size, and eventually generates free oil on the top of O/W emulsions and causes the separation of water at the bottom of W/O emulsions (McClements, 2005).

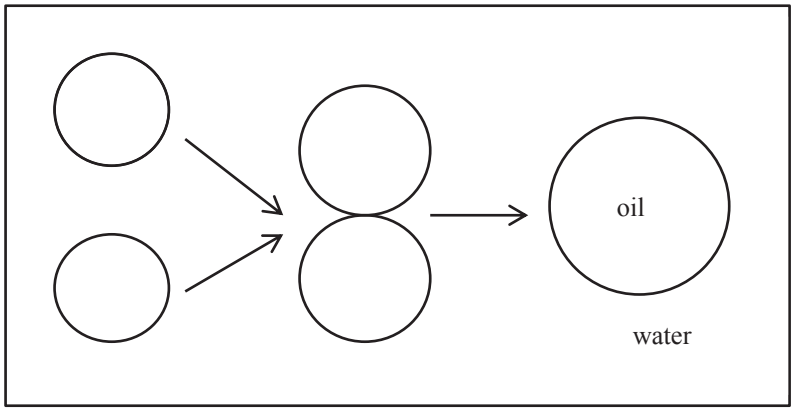


Figure 2.4 Coalescence in an oil-in-water emulsion.

Coalescence can occur only when the droplets come into close contact with each other and the interfacial membranes are disrupted by merging two or more droplets.

Consequently, as described above, it causes emulsion droplets to cream or sediment more rapidly due to its size increase (McClements, 1999, Tcholakova et al., 2006). The larger the contact area between two emulsion droplets leads to an increase in the coalescence rate (McClements, 1999). Coalescence largely relies on short-range forces (e.g. electrostatic and steric interactions) due to the fact that droplets must be in close contact. In general, the susceptibility of droplets to coalescence is determined by the nature of forces acting on between droplets and the resistance of droplet membrane to rupture, and these physical mechanisms are highly dependent on the nature of emulsifiers used to stabilize the emulsion system (McClements, 1999).

Many factors were reported to influence coalescence, such as shear forces, close contact for extended periods, and enzymatic hydrolysis. Proteins as emulsifiers stabilize emulsions against coalescence by creating an interfacial layer against rupture. In some studies, protein molecules are known to stabilize emulsions by their effect on the surface forces, such as by modifying the electrical potential of the drop surface and the van der Waals interactions, or by creating a steric barrier to drop-drop coalescence (Tcholakova et al., 2006). The presence of small amounts of surfactants in the aqueous phase is more prone to induce droplets to coalesce during shearing. This is probably because surfactants adsorb to the interface and increase the mobility of proteins, resulting in ease of rupturing the interfacial layer (McClements, 2005).

The stability of emulsions stabilized by small molecule surfactants is largely dependent on their ability to keep droplets apart, rather than the resistance of rupturing the droplet membrane. For example, non-ionic surfactants such as Tweens stabilize emulsions by having polymeric hydrophilic head groups that provide a large steric overlap and hydration repulsion (McClements, 2005).

2.4 Protein emulsifiers and small molecule surfactants

2.4.1 Protein emulsifiers

Proteins as emulsifiers can readily adsorbs at an oil and water interface, and its hydrophilic side is associated with water molecules and hydrophobic side with oil molecules (Dalgleish, 2003, McClements, 2004). By forming a thick and generally charged adsorbed interfacial layer, emulsion droplets can be stabilized by steric and/or

electrosteric repulsion mechanisms (Singh et al., 2009, Van Aken, 2003). Proteins undergo conformational changes when forming the adsorbed layer, and the extent of these conformational changes depends on the molecular flexibility and packing of adsorbed protein molecules (Singh et al., 2009). Proteins consist of many different groups of amino acids (e.g. hydrophilic, hydrophobic, acidic, basic, polar, non-polar, positively or negatively charged, neutral, etc). The number, distribution and localisation of these amino acids determine the conformation, structure and properties of proteins. Caseins have rather flexible random coil structures, unfold rapidly at the interface and may form extended layers up to about 10 nm thick, whereas more rigid globular proteins such as whey protein (e.g. β-lactoglobulin) take much longer to change conformation and unfold their conformational structures to some extent at the surface and form a compact thin layer of about 2 nm (Singh et al., 2009, McClements, 2004, Dalgleish, 2004). Whey protein is a typical compact globular structured protein, with a rather uniform distribution of non-polar, polar, and charged residues. These proteins fold intramolecularly, burying most of their hydrophobic amino acid residues in the interior of their molecules. In this way, extensive self-association between proteins molecules are prevented (McClements, 2005).

2.4.1.1 Sodium caseinate

Caseinate are produced from skim milk by lowering the pH to 4.6 by addition of lactic or hydrochloric acid or by addition of microbial cultures (rennet) to precipitate caseins. The precipitated caseins are separated from the serum phase containing whey proteins, and washed with water, and then re-dispersed in water through neutralization with alkali salts (e.g. NaOH and KOH) followed by spray drying to form caseinate (e.g. sodium caseinate and potassium caseinate) (Dickinson, 1987).

Sodium caseinate is known as an excellent emulsifier, being the most commonly used in food emulsions due to its good solubility, surface activity, heat resistance, and water holding properties (Dickinson, 1999, Srinivasan et al., 2000). When oil or fat is emulsified in the solution of sodium caseinate, caseins orient on the oil-water interface with the hydrophobic part in the oil phase and the hydrophilic part in the water phase. They provide a long-term stability to oil-in-water emulsions by a combination of electrostatic and steric repulsive forces (Eliot and Dickinson, 2003, Ma et al., 2009). A research study shows that sodium caseinate has a great ability to reduce the oxidation

rate of emulsified lipids compared with Tween 20 due to its ability to form a thick interfacial layer around oil droplets (Kargar et al., 2011). Caseinate-stabilized emulsions are found to be more stable to thermal treatment than whey protein-stabilized emulsions, due to the fact that the relatively flexible casein molecules do not undergo appreciable heat-induced conformational changes unlike globular proteins (e.g. whey proteins) do undergo protein denaturation (McClements, 2005) .

The stability and rheology of sodium caseinate-stabilized emulsions mainly depend on two main factors, which are the structure and composition of the adsorbed protein layer at the oil-water interface and the self-assembly and aggregation state of the protein in the continuous phase (Dickinson, 1999). Sodium caseinate consists of four surface-active caseins (α_{s1}-, α_{s2}-, β-, and κ-casein) (Eliot and Dickinson, 2003). In sodium caseinate-stabilized oil-in-water emulsion, all four caseins are found to adsorb at the emulsion droplet surface. However, competitive adsorption of caseins is largely reported to occur at the interface of caseinate-stabilized emulsions and the extent of the competitive adsorption is affected by the total protein content. For example, β-casein is more likely to adsorb when the emulsion is made with low levels of sodium caseinate (<2%), while α_{s1}-casein preferably adsorbs at higher levels of caseinate (Srinivasan et al., 2000). This is probably due to the high hydrophobicity of β-casein compared to the other casein molecules. Therefore, at a low level of caseinate, β-casein has a higher affinity to bind to the oil-water interface. On the other hand, at a higher level of caseinate, β-caseins tend to self-associate due to their high hydrophobicity or interact with other caseins to form associated structures (Lucey et al., 2000). Caseinate fractions enriched with β-casein exhibit improved surface activity and emulsifying capacity but reduced film stability, whereas those enriched with α_s-/κ-caseins show the opposite behavior (Dickinson, 1999).

2.4.1.2 Whey Proteins

Whey proteins are defined as those proteins remaining soluble after precipitation of caseins in milk at the isoelectric point (pI) (i.e. pH 4.6) of caseins (Malaki Nik et al., 2010b, Dickinson, 2010a, Sarkar et al., 2009b). After separation from caseins, whey proteins are produced by concentration and separation from other components using ultrafiltration, diafiltration and ion-exchange techniques followed by spray drying steps. Whey protein concentrates (WPC) and whey protein isolates (WPI) are concentrated

forms of whey proteins (McClements, 2005) with a high level of protein up to 80-95% (Singh, 2005). Whey proteins are also widely used in food applications, due to various food protein functionalities such as gelling, emulsifying and foaming properties.

Among whey proteins in milk, β-lactoglobulin (β-lg) and α-lactalbumin (α-la) are the two major whey proteins found in bovine milk which together make up about 70-80% of the total whey proteins in milk. They are surface active and both adsorb at the oil-water interface (Malaki Nik et al., 2010b). β-lg and α-la are normally found at the interface proportionally to their original concentration in the aqueous phase if whey protein is used as the only emulsifier (Malaki Nik et al., 2010b).

A single polypeptide chain of β-lg consists of 162 amino acid residues and its molecular weight is about 18.3 kDa. It contains one free sulfhydryl group and two disulphide bonds. β-lg (pI is around 5.2) exists in different polymeric states in an aqueous solution, depending on the pH of β-lg dispersion. It exists as a monomer at pH below 3, an octamer at around pH 4.6 and a dimer between pH 5.2 and 7.2 with a molecular weight of 36 kDa (McClements, 2005, Malaki Nik et al., 2010b, Molinari et al., 1996). α-la, which is the second major globular whey protein, has a molecular weight around 14.2 kDa, and contains four disulphide bonds and no free thiol groups (Malaki Nik et al., 2010b, Nakai and Modler, 1996). α-la has an isoelectric point of pH 5.1, and exists as a monomer at this pH and is nearly spherical (Nakai and Modler, 1996).

β-lg dominates the characteristics of whey protein isolates (WPI) due to its relative high concentration and physicochemical properties (Malaki Nik et al., 2010b), This implies that the physicochemical properties of WPI-stabilized emulsions are qualitatively similar to those prepared with β-lg only. Since whey proteins are globular proteins with a well-defined three-dimensional conformation, they are sensitive to heat-induced protein denature. This often results in the protein-protein interactions between denatured proteins by hydrophobic and disulfide interactions. It has been shown that flocculation and aggregation of WPI-stabilized emulsion droplets occur on heating above 70^{o}C at pH 7, especially with the addition of salt, but it is not likely to happen at pH 3 (Dickinson, 2010a). The extent of heat-induced flocculation of WPI-stabilized emulsions has been shown to be affected by a variety of factors including heat treatment temperature and time, emulsion droplet concentration and unadsorbed protein concentration in the

aqueous phase. Flocs are firmly held together by a combination of hydrophobic interactions and covalent disulphide bonds between proteins that are adsorbed onto the oil droplets (Dickinson, 2010a). α-la protein itself is less heat sensitive, in terms of its interaction with other denaturated proteins, due to the lack of thiol groups, however, together with β-lg, sulfhydryl-disulfide intermolecular reactions can be easily induced, thereby promoting the instability of WPI emulsions to thermal treatment (Nakai and Modler, 1996, Malaki Nik et al., 2010b).

2.4.2 Small molecule surfactants

Surfactants have a hydrophilic "head" group that is attached to a hydrophobic "tail" group, thereby they can stabilize emulsions by forming an interfacial layer around oil droplets with the head group in water and the tail group in oil. The molecular weight of surfactants is small relative to the protein or polysaccharide emulsifiers (e.g. sodium caseinate, WPI, gum Arabic), hence they are often referred to as small molecule surfactants. They form relatively thin and mobile adsorbed layers and the thickness of the thin film is stabilized by lateral diffusion of the molecules using the Ewers and Sutherland mechanism (Van Aken, 2003, Clark et al., 1990). Surfactants are characterised largely by the nature of their head and tail groups. The head group may be anionic, cationic, zwitterionic or non-ionic, and the tail group normally consists of one or more hydrocarbon chains with 10-20 carbon atoms attached in each chain. Both ionic and non-ionic surfactants are widely used in food emulsions, ionic surfactants, such as lecithin, fatty acid salts and citric acid esters of monoglycerides (MG), and non-ionic surfactants, such as polysorbates, acetic acid esters of MG and monoglycerides. Small molecular weight surfactants are more effective in their ability to reduce the interfacial tension during the emulsification of lipids or oils in water in comparison with protein-based emulsifiers (Dickinson, 1997). Non-ionic surfactants normally stabilize emulsion by a combination of steric, hydration, and thermal fluctuation interactions (McClements, 2005). Therefore, they are less sensitive to pH and ionic strength and they do not usually bind to highly hydrated ions. However, the interfacial membranes formed by non-ionic surfactants are prone to break down when droplets come into close contact because of no electrostatic repulsion acting on between droplets (McClements, 2005, Dimitrova and Leal-Calderon, 1999). The stability of emulsions stabilized by small molecule surfactants to aggregation and coalescence largely depends on the ability to keep the droplets apart rather than the resistance of the droplet membrane to rupture

(McClements, 2005). Small molecule surfactants are also likely to self-assemble in aqueous solutions to form spherical micelles or vesicles, depending on their concentration (Dimitrova and Leal-Calderon, 1999).

2.4.2.1 Tween 20

Tween 20, also known as polysorbate 20, is the commercial trade name for the polysorbate surfactant. It is one of the small molecule surfactants commonly used as emulsifiers. Chemically, it is polyoxyethylene (20) sorbitan monolaurate and is a non-ionic surfactant with a hydrocarbon tail containing 12 carbon atoms and a polar head group consisting of three polyoxyethylene chains (Chanamai et al., 2002). Tween 20 has a very low critical micelle concentration at around 2 x 10^{-5} mol/L (Dimitrova and Leal-Calderon, 1999). A schematic chemical structure of Tween 20 is shown in Figure 2.5.

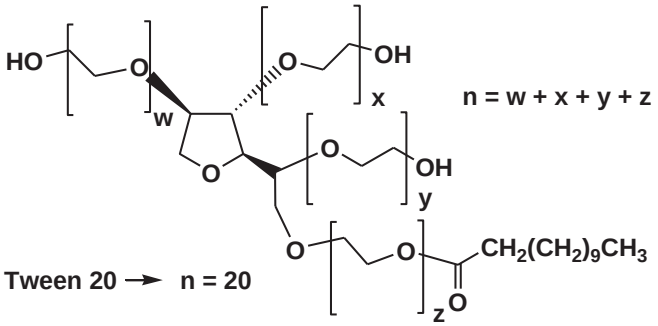


Figure 2. 5 Chemical structure of Tween 20 (Di Marzio et al., 2011).

For emulsions stabilized with Tween 20 alone, the classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory was observed. At high levels of Tween 20 concentrations, because of micellar condensation on the droplet surfaces, a combined effect of the classical DLVO behaviour (van der Waals attraction and the electrostatic repulsion) with an additional steric-like effective repulsion was observed (Dimitrova and Leal-Calderon, 2000). Tweens prevent the occurrence of coalescence of emulsion droplets by a large steric overlap and hydration repulsion provided by polymeric hydrophilic head groups (McClements, 2005).

2.5 Factors affecting emulsion stability

The stability and properties of emulsion systems have been shown to be affected by various environmental factors, such as pH, salts, ionic strength, temperature and freezing.

2.5.1 pH

Most emulsion systems stabilized by protein emulsifiers are negatively charged at neutral pH. The degree of surface electrical charge of emulsion droplets varies with types of proteins employed in the preparation of emulsions. The mechanism for the stability of protein-stabilized emulsions against droplet flocculation is mainly driven by electrostatic repulsion rather than steric repulsion. The stability of emulsions stabilized with small molecule non-ionic surfactants is however driven by steric repulsion (McClements, 2004). This implies the emulsions stabilized with proteins are sensitive to pH and ionic strength (McClements, 2004). Emulsion droplets tend to flocculate at pH around pI of the adsorbed protein because the electrostatic repulsion between droplets is no longer strong to overcome the various attractive interactions such as van der Waals and hydrophobic interactions (McClements, 2004, Dickinson, 2010a).

In case of an emulsion stabilized with sodium caseinate, at neutral pH 7, the adsorbed α_{s1}- and β-caseins mainly contribute to the emulsion stability by providing the net negative charges to the droplet surfaces, thus conferring electrostatic repulsive force between the droplets and making them repel each other (McClements, 2005). However, when the pH is lowered to the protein's pI, the electrostatic stabilizing inter droplet repulsion is no longer available due to a loss of electrical net negative charge (i.e. net zero charge). This induces droplet flocculation and eventually leading to aggregation of droplets (Eliot and Dickinson, 2003, Dickinson, 2010a).

A study has been done to find out the effects of pH 3-7 and WPI concentrations (0.5-2.5%) on the properties of WPI- stabilized emulsions containing 20 wt% soy oil (Hunt and Dalgleish, 1994). The emulsions were the most stable at pH 7 and at pH 5.5 being the least. At pH 7, β-lg and α-la adsorbed proportionally to their concentration, while α-la was found to adsorb preferentially at lower pH values.

2.5.2 Ionic strength

Emulsion droplets tend to flocculate when ionic strength exceed a certain level (McClements, 2004). Therefore, the effect of an addition of electrolytes, such as monovalent and divalent ions, into the aqueous phase of emulsions has raised great interest among scientists. Many publications have explained the effect of Na^+ (Hur et al., 2011a, Srinivasan et al., 2000, Djordjevic et al., 2004) and Ca^{2+} (McClements, 2004, Dickinson, 2010a, Agboola and Dalgleish, 1995, Keowmaneechai and McClements, 2002, Ye and Singh, 2000, Dickinson and Galazka, 1991) on different types of emulsions.

2.5.2.1 Monovalent ions

Monovalent ions, such as Na^+ and K^+, are found to promote emulsion instability due to reduction in the electrostatic repulsive force between droplets by its adsorption onto the emulsion droplet surface via ionic interactions, therefore, the zeta potential (e.g. electrical charge) of emulsion droplets is reduced by its screening effects (McClements, 2004).

A study by Djordjevic et al. (2004) reported the influence of NaCl on the physical properties of WPI-stabilized emulsions. The results showed that at NaCl concentration greater than 150 mM, the emulsions had significant increases in the mean particle size, apparent viscosity, and creaming instability due to flocculation and coalescence of emulsified oil droplets induced by screening of the electrostatic repulsion between droplets (Djordjevic et al., 2004).

Dickinson et al. (1998) showed that emulsions stabilized by β-casein or sodium caseinate were stable at the ionic strengths of 10 - 200 mM NaCl at pH 5.5 and 7. Emulsions stabilized by α_{s1}-casein were flocculated at an ionic strength of 100 mM NaCl or above and the degree of flocculation increased as the pH decreased (Dickinson et al., 1998).

Srinivasan et al. (2000) reported that the addition of NaCl into sodium caseinate-stabilized emulsions after emulsification had an influence on the surface protein coverage and composition. In emulsions prepared with 1% caseinate, the addition of 40mM NaCl after emulsification increased the surface protein coverage by up to 70%

over that for the control emulsion eventually increased the stability of emulsions against droplet flocculation. This appeared to be due largely to the enhanced adsorption of α_{s1}-casein at the droplet surface. For emulsions with 3% caseinate, there was extensive flocculation of emulsion in the absence of NaCl, whereas the addition of NaCl up to 200 mM decreased the extent of flocculation and improved the creaming stability. The authors have reported the possible reason for this was because the increase of surface protein coverage resulted in an increase in adsorbed protein and thus the remaining unadsorbed protein concentrations were not able to reach the critical flocculation concentration (described under section 2.3.1) and therefore could not induce depletion flocculation. In this way, the emulsion stability is enhanced (Dickinson and Golding, 1997, Srinivasan et al., 2000).

2.5.2.2 Divalent ions

Divalent ions, such as Ca^{2+} and Fe^{2+}, promote emulsion flocculation due to their screening electrostatic and binding effects. They reduce the zeta-potential by lowering surface charge density (McClements, 2004, Agboola and Dalgleish, 1995, Dickinson, 2010a). Multivalent counter ions have been found to increase the emulsion instability more effectively than monovalent ions as they are more effective in screening electrostatic interactions (reducing the Debye screening length). They could bind to chelating agents or biopolymers or droplet surfaces, thereby, reducing the zeta potential and inducing the droplets aggregation (Keowmaneechai and McClements, 2002, McClements, 2004). Ca^{2+} bind to the surface of caseinate-stabilized emulsions because of the presence of phosphoserine residues of caseins on casein-coated droplets, while it binds to whey proteins such as β-lg by electrostatic reactions with ionic amino acid groups of the polypeptide chains (Agboola and Dalgleish, 1995, Keowmaneechai and McClements, 2002).

Agboola et al. (1995) has reported the calcium induced destabilization of the O/W emulsions stabilized by 0.3-2% caseinate or β-lg (Agboola and Dalgleish, 1995). They have found that the emulsion stability increased with the amount of protein and decreased with an increase of calcium concentration, however, the destabilization could be reduced with the addition of 50 and 100 mL NaCl to both emulsion systems. The addition of calcium into emulsion a system was reported to destabilize emulsions systems, which was due to the bridging effects induced droplet aggregation (Agboola

and Dalgleish, 1995, Keowmaneechai and McClements, 2002). The authors have attributed the strengthen of sodium caseinate stabilized emulsion with the addition of Na^+ into system to the competition of Ca^{2+} and Na^+ for specific binding sites on phosphoserine groups of caseins, thereby reducing any destabilizing specific effects of Ca^{2+}. Whereas, in the case of β-lg, the possible reason was that cations were competing for negative groups and any specific metal binding sites of polypeptide chains in β-lg solutions (Agboola and Dalgleish, 1995).

Another study showed the effect of addition of $CaCl_2$ on WPI-stabilized emulsions at different pHs (Kulmyrzaev et al., 2000). At pH 5 around the pI of whey proteins, droplets aggregation occurred, regardless of with and without the addition of different concentrations of $CaCl_2$, due to the decreased electrical charge leading to the reduced electrostatic repulsive force between the droplets. At pH 7, the addition of > 3 mM $CaCl_2$, extensive droplet aggregation, viscosity enhancement and creaming instability occurred, whereas these effects were reduced significantly even when a very high concentration of 150 mM $CaCl_2$ was added at pH 3 (Kulmyrzaev et al., 2000).

2.6 In vitro emulsion digestion studies

Food passes through the mouth into the stomach and then the small and large intestines when ingested (Sarkar et al., 2010a). In the mouth, food is exposed to a wide range of physico-chemical processes and changes, such as mixing with saliva and air, heating or cooling to body temperature, changes in ionic strength and pH, deformation during mastication, and exposure to salivary enzymes and biopolymers such as mucin (Sarkar et al., 2009b). Food in the mouth is broken down into smaller pieces by chewing to form a "bolus" (Beysseriat et al., 2006). When bolus is swallowed, it rapidly passes through the mouth to oesophagus and then into the stomach (Sarkar et al., 2009b, Beysseriat et al., 2006). It undergoes a drastic change by the pH and mechanical agitation due to peristaltic movement in the stomach. Partially digested foods encounter a broad range of physicochemical conditions when pass through the stomach to small intestine, including mixing with different types of enzymes (trypsin, chymotrypsin and lipases), coenzymes (co-lipases), and surface active agents (bile salts and phospholipids) (Singh et al., 2009).

The physico-chemical changes occurring in emulsions during their passage through the gastrointestinal (GI) tract are not clearly understood due to the complexity of food systems and GI systems. In recent years, a number of studies have been conducted to understand the digestion behaviour of different types of emulsions using *in vitro* models simulating the physiological conditions of GI tract, such as simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) containing enzymes and bile salts. However, it should be noted that *in vivo* conditions can never be completely simulated under *in vitro* conditions, therefore, the results of *in vitro* studies of emulsion digestion may not correlate to the *in vivo* emulsion digestion (Boisen and Eggum, 1991). Several advantages of *in vitro* studies over *in vivo* studies are that it is less time consuming, less expensive, more reproducible, and easy to perform and that it allows the simple methods for comparing the digestion behaviour of emulsions with different composition and structure stabilized by different types of emulsifiers. Nevertheless, in designing *in vitro* digestion models, there are a number of factors that should be considered, including sample characteristics, enzyme activity, ionic composition, applied mechanical stresses and digestion times all of which have significant influences on the *in vivo* digestion of emulsions.

The characteristics of emulsion type and composition and enzyme type and concentration are some of the key factors that affect the digestion of emulsions during *in vitro* digestion. A number of studies have found that gastric enzymes and pancreatic lipases induce emulsion droplet flocculation and coalescence (Mun et al., 2007, Van Aken, 2003, McClements, 2004, Vingerhoeds et al., 2005, Surh et al., 2006). Enzymes such as lipases are present in the stomach (gastric lipase) and pancreas (pancreatic lipase), and they adsorb to the surfaces of emulsified lipids and hydrolyze emulsified triacylglycerols into diacylglycerols, monoacylglycerols and free fatty acids. Proteases present in the stomach (pepsin) and small intestine (trypsin and chymotrypsin) are responsible for breaking down proteins adsorbed at the interface of lipid droplets into small peptides and amino acids, thus causing the physicochemical changes in emulsion droplets (Hur et al., 2011b). The pepsin to substrate ratio that has been used in most *in vitro* studies varies from 1:3 to 1:10, which greatly exceeds the actual quantity found in the human stomach, even in fed state conditions (Wickham et al., 2009). In addition, gastric lipase has not been used in the studies even gastric lipases are normally present at a level of 0.5-1.0 μM in the human stomach which may be due to its little lipolysis

activity in the overall lipid digestion in healthy human adults. The gastric lipase activity however is significant only for individuals suffering from pancreatic lipase insufficiency (Singh et al., 2009, McClements et al., 2009a).

2.7 Gastric digestion

2.7.1 Acidic pH and ionic strength

Typically, the pH of the fasting human stomach ranges from 1-3 (Sarkar et al., 2009b, Hur et al., 2009, Singh et al., 2009, McClements et al., 2009a), and the ionic strength is around 100 mM (McClements et al., 2009a). However, the pH varies depending on the quantity and nature of the ingested food and also differs among individuals (McClements et al., 2009a). Generally, there is a significant increase in the pH of the stomach contents after consuming a food, followed by a gradual decrease to the highly acidic value of the stomach in a fasted state, which is due to the stimulated secretion of gastric acid. The time taken to return to acidic pH varies depending on the initial pH, buffering capacity, composition, and quantity of food ingested (Kalantzi et al., 2006, McClements et al., 2009a). The amount of food remaining in the stomach after ingestion decreases by about 50% in 30-90 minutes, and liquid components digested move more rapidly than solid components (McClements et al., 2009a). The acidic conditions of the stomach take part in many roles, such as activating enzymes, hydrolysis of food components, and killing of bacteria (Kalantzi et al., 2006, McClements et al., 2009a). It has been reported that most of milk-protein-stabilized emulsions undergo substantial changes in droplet characteristics during this gradual decrease in pH from neutral to acidic conditions because of full charge reversal as well as some possible aggregation effects around the p*I* (around 4.5-5.2) (Singh et al., 2009).

In the fasted state, the osmolality and ionic strength in the stomach are around 190 mOsm/kg and 100 mM, respectively. The concentrations of major ions are Na^+: 70 ± 30 mM, K^+: 13 ± 3 mM, Ca^{2+}: 0.6 ± 0.2 mM, and Cl^-: 100 ± 30 mM (McClements et al., 2009a). The osmolality and ionic strength of the stomach contents increase significantly after ingestion of food, which is due to the additional ions and other solutes derived from the food (Kalantzi et al., 2006). Kalantzi et al. (2006) has reported that the osmolality of stomach increases drastically from ~ 140 mOsm/kg in the fasted state to ~

560 mOsm/kg after ingestion of a nutrient drink (Kalantzi et al., 2006). High ionic strengths might contribute to electrostatic changes in the protein-stabilized emulsions leading to salt-induced aggregation of emulsion droplets. Therefore, the drastic change in pH and ionic strength in the stomach could result in changes in emulsion droplets by flocculation or change in the interfacial layers.

2.7.2 Pepsin

Pepsin is a protease present in the stomach, and is responsible for breaking down proteins and peptides into smaller peptides and amino acids (Hur et al., 2011b). The hydrolysis of protein is initiated by the action of pepsin and acid. At least half of the proteins leaving the stomach are usually in the form of peptides (Boisen and Eggum, 1991).

The protein-stabilized emulsion is altered into a cationic form as the pH of the stomach is lower than the pI of proteins (pH 4.5-5.2 for casein or whey protein). In addition, the emulsion is mixed with gastric secretions containing proteolytic and lipolytic enzymes and minerals (Sarkar et al., 2009b, Singh et al., 2009, Beysseriat et al., 2006). In the presence of pepsin in the stomach, the proteolytic action on the adsorbed protein reduces the ability of protein from its protection to emulsion, and removes the droplet charge and steric repulsion barriers. As a result, aggregation and coalescence of emulsion droplets can occur. The presence of salts in the gastric juices also promotes droplet flocculation due to electro screening effects (Sarkar et al., 2009b, Singh et al., 2009, Armand et al., 1999).

Several studies have shown that caseins were very susceptible to proteolysis by pepsin in its native state due to their flexible random coil structure. However, whey proteins, such as β-lg, were very resistant to the acids and proteolytic enzyme (pepsin) present in the stomach due to its compact structure but other whey proteins, such as α-la, were shown to be hydrolysed by pepsin (de Wit, 1998, Dalgalarrondo et al., 1995, Reddy et al., 1988, Malaki Nik et al., 2010b, Kim et al., 2007). However, β-lg as emulsifier in o/w emulsions adsorbing onto the oil droplets was shown to be hydrolyzed by pepsin during *in vitro* gastric digestion. The increased digestibility of β-lg to pepsin was explained to be due to its unfolding at the emulsion interface that exposed the peptic cleavage sites (Sarkar et al., 2009b). Interestingly, it was shown that α-la adsorbed at the

interface was resistant to hydrolysis by pepsin. Van Aken et al. (2007) examined differences in the *in vitro* gastric digestion behaviours of emulsions stabilized by Tween 80, whey protein, or whey protein and caseinate. The emulsions stabilized by Tween 80 showed the high emulsion stability against flocculation and coalescence compared with the others as Tween 80 is a non-protein, non-ionic emulsifier, thus relatively stable to low pH and enzymes (van Aken et al., 2011).

2.8 Intestinal digestion

Partially digested foods in the stomach further encounter a broad range of physicochemical conditions when they pass through the stomach to small intestine, including mixing with different types of enzymes (trypsin, chymotrypsin and lipases), coenzymes (co-lipases), and surface active agents (bile salts and phospholipids) (Singh et al., 2009). It will also undergo a drastic change in the pH and ionic strength as a result of the secretion of electrolytes by the pancreas (Singh et al., 2009).

2.8.1 pH and ionic strength

The pH increases from highly acidic (pH 1 to 3) in the stomach to around neutral (6-7.5) in the duodenum when the ingested food passages through the stomach into the intestine, where the digestion of foods occurs by pancreatic enzymes (McClements et al., 2009a, Hur et al., 2009, Golding and Wooster, 2010). The osmolality of duodenum contents in the fasted state is around 180 mOsm/kg and the ionic strength of small intestine is around 140 mM (Kalantzi et al., 2006, McClements et al., 2009a). There is a significant increase in the osmolality and ionic strength after ingestion of food due to the additional ions and other solutes arising from food (McClements et al., 2009a). The osmolality has been reported to increase from ~180 mOsm/kg in the fasted state to ~ 290 mOsm/kg after ingestion of a nutrient drink (Kalantzi et al., 2006). The ionic strength is very important in the small intestine since it influences the electrostatic interactions in the system. Some type of ions, such as divalent cations, may promote precipitation of bile salts and long chain statured fatty acids in the small intestine, therefore decreasing their digestibility (McClements et al., 2009a).

2.8.2 Bile salts

Bile salts present in the small intestine are synthesized from cholesterol in the liver (Beysseriat et al., 2006, McClements et al., 2009a). The “backbone” of bile salt

molecules is cholic acid (water insoluble), which is conjugated with amino acids (taurine or glycine), thus conferring its water solubility (McClements et al., 2009a). Bile salts have a highly rigid "plate-like" molecular structure with a hydrophobic side and a hydrophilic side. Bile salts are natural biosurfactants in the gut lumen and very important in both the digestion and adsorption of lipids (Golding and Wooster, 2010).. The hydrophobic side can interact with substances that have a non-polar character whereas the hydrophilic side can interact with substances that have a polar character (McClements et al., 2009a). Bile salts may adsorb directly to any freshly created oil-water interface or they may displace the surface active materials present at oil-water interface, thereby altering interfacial composition and properties (Hur et al., 2009). In the case of emulsions, bile salts displace other surface active materials from the surface of emulsion droplets and they provide access for pancreatic lipase to the emulsified lipid core (Sarkar et al., 2009c, Singh et al., 2009). Therefore, they promote the binding of lipase to the interfacial layer and accelerate the lipid digestion (Singh et al., 2009). In this aspect, bile salts play a very important role in many different activities, including facilitation of lipid droplet deformation or stabilization of droplets against aggregation and formation of micelles that solubilize and transport hydrophobic materials (McClements et al., 2009a).

2.8.3 Pancreatic lipase

Gastric lipase induces the limited hydrolysis of lipids in the healthy human adult's stomach and more substantial levels of lipolysis occur in the small intestine by pancreatic lipase (Sarkar et al., 2010a). The lipids (e.g. triglycerides) contained within emulsion droplets is hydrolyzed by pancreatic lipase into monoglycerides and fatty acids (Boisen and Eggum, 1991). The pancreatic lipase gets activated when it reaches the oil-water interface where it hydrolyses the fatty acids of triglycerides at sn-1 and sn-3 position, resulting in the production of sn-2 monoglycerides and free fatty acids (Hur et al., 2009). This means that before pancreatic lipase can hydrolyze the emulsified lipids, it is necessary for it to adsorb to the surfaces of the emulsion droplets. The binding of pancreatic lipase at the droplet surfaces depends on the presence of co-lipase, bile salts and calcium (Hur et al., 2011b) and the "quality" of interface including the chemical composition and physical structure of the interfacial layer (McClements et al., 2009a).

The presence of bile salts promotes the displacement of other surface active materials (e.g. proteins) at the interface, providing access for pancreatic lipase to the emulsified lipid core. However, it should be noted that it may inhibit the pancreatic lipase activity on the basis of the concentration of bile salts. Lipase activity is likely to be promoted at relatively low concentrations of bile salts, which mainly attribute to their ability to solubilize lipid digestion products and remove them from the oil-water interface, such as sn-2 monoglycerides and free fatty acids. On the other hand, the ability of lipase to digest emulsified lipids may be inhibited at relatively high bile salt concentration because bile salts compete for the oil-water interface with the lipase, thus preventing it from coming into close proximity to the lipid substrate (McClements et al., 2009a).

2.9 Conclusions

Recently, the digestion of emulsions has raised great interest among scientists. Many studies have been carried out to understand the mechanisms of emulsified lipid digestion and absorption in the gastrointestinal tract using *in vitro* models such as simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). However, most studies have focused on the emulsion digestion in either SGF or SIF, and the sequential digestion in both SGF and SIF was studied to a limited extent. The objective of this study was to examine the digestion behaviour and hydrolysis of lipids of emulsions occurring during the intestinal digestion after the gastric treatment. For this study, three different types of emulsifiers, commonly used in the food industry were used to prepare O/W emulsions, including sodium caseinate, whey protein isolate (WPI) and non-ionic surfactant Tween 20.

Chapter 3. Materials and Methods

3.1 Materials

In this study, three different types of emulsifiers, such as whey protein isolate (WPI), sodium caseinate, and Tween 20, were used in the preparation of oil-in-water emulsions. WPI (ClearProtein™ 895 WPI) was supplied by Fonterra Co-operative Group Ltd. (Palmerston North, New Zealand). As stated by the manufacturer, the composition of the WPI powder was 92.0% protein, 4.8% moisture, 0.4% fat, 0.5% carbohydrate and 2.3% ash. Sodium caseinate (Alanate 180) was obtained from Fonterra Co-operative Group Ltd. (Palmerston North, New Zealand). The composition of the sodium caseinate powder was 92.7% protein, 4.3% moisture, 0.7% fat, 0.2% carbohydrate and 3.6% ash. Tween 20 (polyoxyethylenesorbitan monolaurate, CAS: 9005-64-5, P7949-500mL) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). It was a clear light yellow to yellow-green viscous liquid and contained ≥40% lauric acid and ≥80% fatty acid (sum of lauric, myristic, palmitic and stearic acids). Soy oil containing 92g/100 mL fat (Soy Premium Quality, Goodman Fielder Home Ingredients NZ Ltd., Auckland, New Zealand) was purchased from a local supermarket.

All enzymes and porcine bile extract used for the study of the *in-vitro* gastrointestinal (GI) digestion of emulsions were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Pepsin (EC 232-629-3, P7000) from porcine gastric mucosa contained 345 units/mg solid and 863 units/mg protein. Pancreatin (EC 232-468-9, P7545) from porcine pancreas (4 x USP specifications) contained amylase, lipase, trypsin, ribonuclease and protease. Bile extract porcine (EC 232-369-0, B8631) contained glycine and taurine conjugates of hyodeoxycholic acid and other bile salts.

Oleic acid (purity > 99%; Product No: O1008; CAS No: 112-80-1) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and used as a standard fatty acid for determination of free fatty acids hydrolyzed from emulsified lipids during digestion of emulsions. Milli-Q water purified and deionised by treatment with a Milli-Q apparatus (Millipore Corp., Bedford, MA, USA) was used for the preparation of all solutions. All other chemicals used were of analytical grade obtained from either BDH Chemicals (BDH Ltd., Poole, England) or Sigma Chemicals Co. (St. Louis, MO, USA).

3.2 Emulsion Preparation

Oil-in-water (O/W) emulsions (1.0 wt% emulsifier and 20.0 wt% soy oil) were prepared using WPI, sodium caseinate or Tween 20 as emulsifiers to investigate the digestion behaviour of emulsions stabilized by different types of emulsifiers under the simulated GI conditions. Aqueous solutions of emulsifiers (1.25 wt%) were prepared by dispersing an emulsifier in Milli-Q water and stirring for at least 2 hrs gently at 20^{o}C for the complete dissolution and hydration. Sodium azide (0.02 wt%) was added as an antimicrobial agent to prevent the microbial growth.

Emulsions were prepared by a two-step process. In the first step, 20 wt% soy oil and 80 wt% aqueous emulsifier solution were mixed to form a coarse emulsion by using a conventional rotor-stator high shear mixer (Silverson L4RT, OFI Testing Equipment, Inc, Huston, USA) at 6,500 rpm for 3 minutes. In the second step, the coarse emulsion was homogenized 4 times using a two-stage high pressure homogenizer (12.5H, RANNIE, Denmark), as shown in Figure 3.1, operated at 250 bar (25 MPa) and 50 bar (5 MPa) in the first and second stages, respectively, to form a fine emulsion. An ice bath was used to prevent protein denaturation during the homogenization process.

Figure 3.1 Two-stage high pressure homogenizer.

3.3 Simulated Gastric and Intestinal Fluids

Simulated gastric fluid (SGF) containing 34 mM NaCl was prepared by adding 2 g of NaCl and 7 mL of 1.0 M HCl in water. After adjusting the pH to 1.6 using 1.0 M HCl, the final volume was made to 1 L with distilled water. The SGF digestion of emulsions was carried out with or without the addition of pepsin. The amount of pepsin added was 3.2 mg per mL of the final mixture of emulsion and SGF. The enzyme pepsin in a power form was added directly to the emulsion/SGF mixture. Simulated intestinal fluid (SIF) consisted of 39 mM K_2HPO_4, 150 mM NaCl and 30 mM $CaCl_2$ and the pH was adjusted to 7.5. Bile extract was added at the concentration of 5.0 mg/mL. The enzyme pancreatin (1.6 mg/mL) in a powder form was added immediately after mixing SIF with emulsion (Pharmacopeia, 1995).

3.4 In Vitro Digestion of Emulsions with SGF, SIF and SGF/SIF

For the *in-vitro* gastric digestion of emulsions, freshly prepared emulsions (~pH 6.7-7.2 depending on type of emulsifiers used) containing 20 wt% soy oil were mixed with SGF (pH 1.6) at a 1:1 ratio. This rendered the emulsion oil concentration as 10 wt% oil in the emulsion-SGF mixture. The mixture was incubated for 2 hrs at 37^{o}C in a temperature controlled shaking water bath (Model: BS-11, Biostrategy, NZ) with a shaking speed of 95 rev/min. The pH was monitored and maintained at pH 1.6 using 1.0 M HCl.

For the *in-vitro* intestinal digestion with SIF, freshly prepared emulsions were diluted with distilled water at a ratio of 1:1 rather than directly mixing with SIF in order to keep the oil concentration same as the emulsion/SGF/SIF mixture used for a sequential GI digestion. The diluted emulsion was then added into SIF at a 1:1 ratio which resulted in the final oil concentration as 5 wt%. The emulsion-SIF mixture was then incubated and digested for 2 hrs at 37^{o}C while maintaining the pH 7.5 using 1.0 M NaOH. During the digestion of emulsions with SGF or SIF, aliquots of samples were periodically withdrawn for analysis from the digestion medium and heat-treated immediately at 80^{o}C for 5 mins to inactivate the enzymes.

For the digestion of emulsions sequentially in SGF and SIF, the freshly prepared emulsions were firstly mixed with SGF at a ratio of 1:1 and then incubated for 2 hrs

under the same condition as described above. During the gastric digestion, the emulsion/SGF mixture was withdrawn at different time intervals (1 min, 30 min, 1 hr and 2 hr), transferred into SIF at a 1:1 ratio for incubation at 37^{o}C for 2 hrs. Aliquot amounts of samples were also collected during digestion and analysed for the characterization of emulsion properties (e.g. size, zeta potential, SDS-PAGE and confocal laser scanning microscopy). In this study, the effect of enzymes (pepsin) was also investigated by incubating the emulsions in SGF with or without the addition of pepsin. A schematic diagram of the emulsion digestion processes in SGF or SIF and sequentially in both SGF and SIF is illustrated in Figure 3.2.

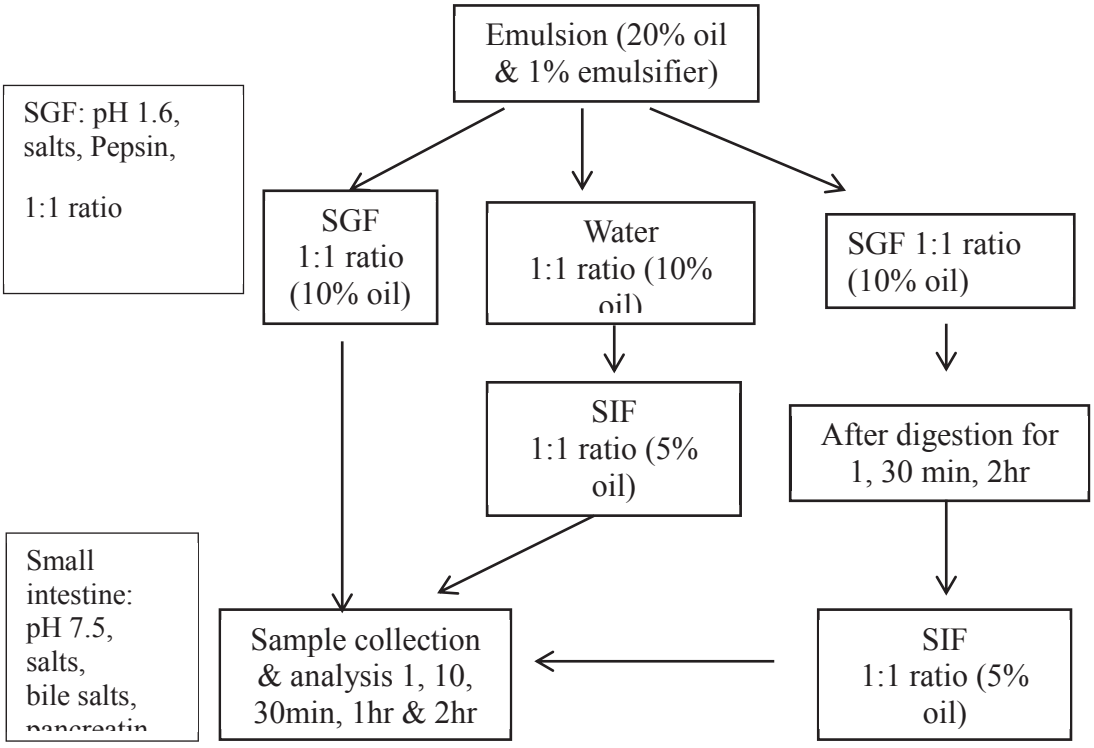


Figure 3.2 Schematic representation of sequential digestion of emulsions in SGF and SIF.

3. 5 Analyses of Emulsions

3.5.1 Particle size and size distribution

The mean particle size and size distribution of emulsions were measured immediately after the emulsion preparation and during an *in-vitro* simulated gastric and intestinal digestion by a static light scattering technique using a particle size analyser (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK) (Figure 3.3). The relative refractive indices for the measurement of emulsion droplet size used were 1.46

for dispersed phase (soy oil) and 1.333 for continuous phase. The absorbance value of emulsion droplets used was 0.001. The size of particles measured were reported as the volume weighted mean diameter $d_{4,3}$ (μm) and surface mean diameter $d_{3,2}$ (μm) which were calculated by the following equations $d_{4,3} = \sum \frac{nidi^4}{nidi^3}$, where *ni* is the number of particles and *di* is the diameter of emulsion droplets, and $d_{3,2} = \sum \frac{nidi^3}{nidi^2}$.

Figure 3.3 Malvern Mastersizer 2000.

3.5.2 ζ-potential

Zeta (ζ) potential of emulsions was determined by a combination of laser doppler velocimetry and phase analysis light scattering (PALS) technique using Malvern Zetasizer Nano ZS (ZEN 3600, Malvern Instruments Ltd., Malvern, Worcestershire, UK) (Malaki Nik et al., 2010a). The measurement of ζ-potential is conducted by applying an electric field across a dispersion system (e.g. emulsions) which causes particles within the dispersion to move towards the oppositely charged electrode at a certain velocity. These movements cause the frequency shift of an incident laser beam, which is measured as electrophoresis mobility (U_E). U_E is converted to zeta potential (ζ) by Henry's equation as following: $U_E = \frac{2\varepsilon\zeta f(Ka)}{3\eta}$, where, ε is the dielectric constant of the medium, η is viscosity, and *f*(*ka*) is the Henry's function that takes the value as 1.5 (Smoluchowski approximation) which is generally used for emulsions having an aqueous phase as the dispersant.

Prior to the measurement, all emulsion samples were diluted 100 times with a medium same as the continuous phase of emulsions (e.g. water, SGF or SIF) to prevent the multiple light scattering effect. The diluted samples were placed into a disposable folded capillary cell equipped with two platinum electrodes at both sides. The temperature of the electrophoresis cell was maintained at 25^{o}C by the Peltier system. The zeta potential was calculated from mean and standard deviations on the basis of running at least 12 times.

3.5.3 Confocal laser scanning microscopy

Microstructures of emulsions during digestion with simulated GI fluids were analysed using a confocal laser scanning microscopy (CLSM) (Leica SP5 DM6000B, Leica microsystems, Heidelberg, Germany) equipped with a motorized z-focus and motorized and coded 7 *x* nose pieces along with three 100 mm oil immersion objectives (Figure 3.4). Nile red (an oil soluble fluorescent dye) was used to stain oil in the emulsions and excited by an argon laser with an excitation line of 488 nm while fast green (a water soluble fluorescent dye) excited with 633 nm helium laser was used to stain protein in the emulsions. A 1 mL of emulsion samples was taken into an eppendorf tube and mixed with 20 uL of nile red and 20 uL of fast green. Samples were stained for at least 5 minutes. A drop of the stained emulsion sample was placed on a concave confocal microscope slide about 1.2-1.3 mm thick (Sail Brand, China) and then covered with a cover slip. A magnification lens at 63x was used to determine the microstructure of emulsions. The position of the confocal plane remained constant during all the measurements.

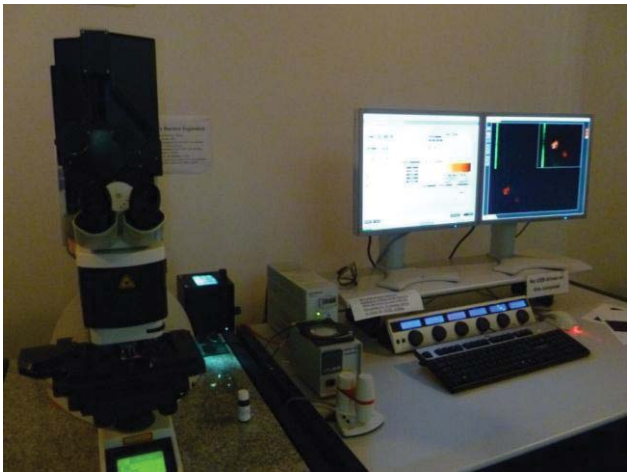


Figure 3.4 Laser Scanning Confocal Microscopy.

3.5.4 pH-stat titration

The hydrolysis of fatty acids from emulsified lipids during digestion of emulsions in SIF was measured by titration (Sarkar et al., 2010b, Malaki Nik et al., 2010a, Hur et al., 2009, Hur et al., 2011a). The emulsion samples (20 wt% soy oil) that were digested first in SGF for different periods of time were collected and added into SIF (5.0 mg/mL bile salts) at a 1:1 ratio. The emulsion-SGF-SIF mixture (5 wt% oil) in a flask was incubated in a water bath at 37^{o}C for 10 minutes. The system was then adjusted to pH 7.5 using NaOH or HCl solutions followed by the addition of 1.6 mg/mL pancreatin powder. The final composition within the reaction flask was 5 wt% oil, 2.5 mg/mL bile salts, and 0.8 mg/mL pancreatic lipases. A pH-stat automatic titration unit (TitraLab 856, Radiometer Analytical SAS) was then used to monitor the pH to be maintained constant at pH 7.5 by titrating and neutralizing free fatty acids generated by lipolysis with 0.05 M NaOH. The volume of 0.05 M NaOH consumed was recorded and calculated as the amount of free fatty acids hydrolysed from the emulsions.

A series of standard oleic acid solutions containing 0-800 μmol oleic acid were prepared and titrated with 0.05 M NaOH to create a standard curve (see Appendix 2). Briefly, a stock standard oleic acid solution containing 5.9 mM oleic acid (MW=282.47) was prepared by dissolving oleic acid in methanol that was pre-adjusted to pH 7.5 using 0.05 M NaOH. Different volumes of the stock standard oleic acid solution were then mixed with the pre-adjusted methanol (pH 7.5) and titrated with 0.05 M NaOH to pH 7.5. The amounts of NaOH consumed were plotted as a function of oleic acid concentrations to create the standard curve which was expressed as the molarity of oleic acid (μM) versus the amount of 0.05 M NaOH consumed (mL). The volume of NaOH consumed was converted to the amount of free fatty acids as μM free fatty acids per mL of the emulsion based on the created standard curve which was then plotted as a function of digestion time.

3.5.5 Electrophoresis

3.5.5.1 Sample preparation

The emulsions samples (sodium caseinate emulsions and WPI emulsions) collected during digestion for 2 hr with SGF were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Srinivasan et al., 1996, Sarkar et al., 2009b). All chemical reagents and solutions prepared for SDS-PAGE are attached in the

Appendix 1. After collection of emulsion samples (pH 1.6), they were immediately adjusted to pH 7.5 using 5 M NaOH in order to inactivate the enzyme (pepsin). The emulsions were then centrifuged using a high speed centrifuge (F21 High Speed Rotor, Fiber*Lite* Centrifuge/Piramoon Technologies, Inc., CA, USA) at 42,828 x g for 40 minutes at 10^{o}C to separate into the cream and serum phases. The subnatant (i.e. serum phase) was withdrawn from each emulsion using a syringe, filtered using a filter paper and mixed with a sample buffer (Tris-HCl buffer at pH 6.8 containing glycerol, SDS, bromophenol blue and β-mercaptoethanol). The cream phase was also collected and washed two times by redispersion in water and centrifugation. The washed cream was then left on a filter paper to drain and remove water. An aliquot of each of the drained washed cream was mixed with a sample buffer (Tris-HCl buffer at pH 6.8 containing glycerol, SDS, bromophenol blue and β-mercaptoethanol) and vortexed vigorously. The serum and washed cream samples mixed with the sample buffer were then heated at 95^{o}C for 5 minutes and then centrifuged at 4^{o}C for 30 minutes at 16,000 x g. The subnatant was withdrawn to run SDS-PAGE.

3.5.5.2 SDS-PAGE

A 10 µL of each sample and molecular weight markers (10-250 kd) were loaded onto each well in SDS-gels (4% stacking gel and 16% resolving gel). The electrophoresis (Bio-Rad Laboratories, Richmond, CA, USA) was conducted with a voltage of 125 mV for 1 hr until the bromophenol bands reached down to the bottom of the gels. After running the SDS-PAGE, the gels were carefully removed and transferred into a Coomassie Brilliant Blue R staining solution (0.3 wt%, 20% isoproponol and 10% glacial acetic acid) in a plastic container. The container was then placed on a platform rocker and shaken for 30 minutes. After staining, the gels were transferred into an aqueous destaining solution (10% isopropanol and 10% glacial acetic acid) and destained by gentle shaking on the rocker and changing the destained solution periodically with a fresh destaining solution until the gels showed clearly separated bands with no background blue colour. The gels were placed onto a scanner (Scanmaker *i*900, Microtek, Carson, CA, USA) to scan the images of SDS-PAGE gels.

3.5.5.3 Statistical analysis

All experiments were carried out in either duplicate or triplicate using freshly prepared samples. Results are reported as the calculated means and standard deviations.

Chapter 4. In Vitro Gastrointestinal Digestion of Oil-in-Water Emulsions Stabilized by Sodium Caseinate

4.1 Abstract

In this study, in vitro digestion and the physicochemical and microstructural changes of sodium caseinate-stabilized emulsions were examined after the emulsions had been digested in a model simulated gastric fluid containing pepsin for different times. The average size, size distribution, microstructure, proteolysis of interfacial proteins and lipolysis of the emulsion droplets were monitored as a function of digestion time. The emulsion droplets underwent extensive droplet flocculation, with some coalescence together with proteolysis of interfacial proteins, in simulated gastric fluid, resulting in changes in the droplet size and the microstructure of the emulsions. In general, digestion in simulated gastric fluid containing pepsin accelerated coalescence of the emulsion droplets during subsequent digestion in simulated intestinal fluid containing pancreatic lipase. However, the changes in the size, the microstructure and the proteolysis of the interfacial proteins of the emulsions under gastric conditions did not influence the rate and the extent of lipid digestion in the subsequent intestinal environment.

4.2 Introduction

A food product passes through the mouth into the stomach and then into the intestine after it has been ingested. The time for which a food stays in the stomach depends on its nature, *e.g.* chemical composition, shape, size, microstructure, pH, ionic conditions and rheological properties (Armand et al., 1992, Singh et al., 2009). An emulsion undergoes a drastic change in pH, from around pH 7 to pH 1~3, and undergoes mechanical agitation because of peristaltic movement in the stomach. Moreover, an emulsion is mixed with gastric fluid, exposing it to different enzymes (pepsin, gastric lipase) and electrolytes (Na^+, Cl^-, Ca^{2+}, *etc*.) as it enters the stomach (Singh et al., 2009, Dickinson, 2010b, Beysseriat et al., 2006).

Milk proteins are known to be easily hydrolysed because of the relatively open flexible structure such as caseins. Adsorbed proteins may be hydrolysed by the proteolytic action of pepsin (Singh and Sarkar, 2011, Singh et al., 2009), which may lead to changes under gastric conditions in the physicochemical properties of emulsions formed with proteins. When a partially digested emulsion enters the small intestine from the

stomach, it experiences a wide range of physicochemical conditions, including being mixed with various enzymes, such as pancreatic lipases, and surface-active agents, such as bile salts. Moreover, it undergoes a substantial change in pH (from 1.5~3.0 in the stomach to ~6.0−7.5 in the intestine) and varies in ionic strength because of the presence of different electrolytes (Singh et al., 2009, McClements et al., 2008). Therefore, the interfacial composition of an emulsion droplet that encounters during passage through the gastrointestinal (GI) tract can be extremely complex and will depend largely on the concentrations and surface activities of the intestinal components at any given time. Model systems are thus used to understand such complex conditions because they allow the digestion behaviour and the interactions of individual physiological components of an emulsion to be investigated separately.

A number of recent studies have reported the physicochemical changes of emulsions using models of either *in vitro* gastric digestion or *in vitro* intestinal digestion (McClements et al., 2008, Sarkar et al., 2009b, Sarkar et al., 2010b, Duffy et al., 2009, Hur et al., 2009, Golding and Wooster, 2010, Sarkar et al., 2010a, Vingerhoeds et al., 2005, Malaki Nik et al., 2010b, Hur et al., 2011b, Hur et al., 2011a). Mun *et al.* (2007) investigated the intestinal digestion of sodium caseinate, whey protein isolate (WPI) and Tween 20 emulsions. They suggested that the prevention of droplet flocculation and coalescence during hydrolysis was affected by the type of emulsifier, with WPI emulsions being the least stable and Tween 20 emulsions being the most stable. Caseinate-stabilized emulsions were more prone to droplet flocculation than to coalescence, whereas WPI emulsions were very prone to coalescence (Singh and Sarkar, 2011). However, in the sequential processing of an *in vitro* oral-to-gastrointestinal model, Hur *et al.* (2009) reported that the emulsifier that was initially used to stabilize an oil-in-water emulsion had only a limited effect on the microstructural changes that occurred during the digestion of the lipid droplets, even though the physicochemical properties of the emulsion changed markedly from the oral environment to the environments of the stomach and the intestine (Singh and Sarkar, 2011). However, no information on the behaviour of an emulsion during intestinal digestion following gastric digestion for different times is available. In the stomach, it has been known that the emulsions undergo a drastic shift in pH because of the highly acidic gastric environment (pH 1–3) and are also exposed to a proteolytic enzyme (pepsin). As the pH is well below the isoelectric point of most food proteins, most of the emulsions acquire

a net positive charge under gastric conditions. Hence, rather than the initial charge of the emulsifier, it is the nature of the protein coated on the droplet surface and its susceptibility to pepsin that drive the emulsion destabilisation process. As a result of proteolysis of the interfacial protein layer, the peptides generated are not strong enough to provide emulsion stability, thus resulting in coalescence (Singh and Sarkar, 2011). In addition, human gastric fluid contains acid-stable gastric lipase that could act on emulsions, resulting in the generation of fatty acids and monoglycerides from the hydrophobic lipid core. As some of these digestion products are surface-active, they have the potential to displace protein/peptides from the interface of protein-stabilised emulsions. The objectives of this study were to change the physicochemical properties of sodium-caseinate-stabilized emulsion droplets to different extents by digesting them in a gastric environment for various times and then to investigate the changes in their physicochemical properties and their *in vitro* lipid digestion behaviour in an intestinal environment.

4.3 Results and discussion

4.3.1 Digestion of emulsions in SGF

4.3.1.1 Droplet size and microstructure

Freshly prepared sodium caseinate-stabilized emulsion (1.0 wt% protein and 20 wt% soy oil) droplets at pH 7 had an average volume mean diameter ($d_{4,3}$) of about 0.49 μm. On mixing with SGF (pH 1.6) containing pepsin, the emulsion droplet size increased sharply to 1.85 ± 0.08 and 2.44 ± 0.03 μm within the first 1 and 10 min of digestion, respectively, followed by a relatively small and constant increase during further digestion (Figure 4.1). The increase in the emulsion droplet size with increasing digestion time could be due to the hydrolysis of adsorbed proteins at the interfacial layers by pepsin, resulting in droplet flocculation and coalescence (Sarkar et al., 2009a). This hypothesis was able to be confirmed by the fact that there was no significant change in the particle size and size distribution of emulsions when mixed with SGF without added pepsin.

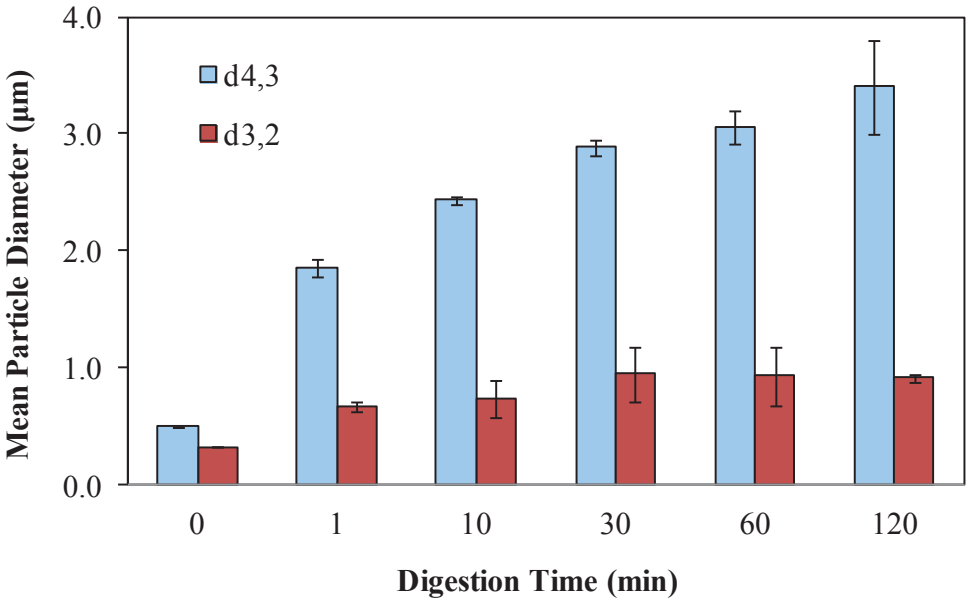


Figure 4.1 The volume ($d_{4,3}$) and surface ($d_{3,2}$) mean diameters of original sodium caseinate-stabilized emulsion (20 wt% soybean oil and 1.0 wt% protein) and of emulsions after digestion in SGF (pH 1.6) containing pepsin for different times.

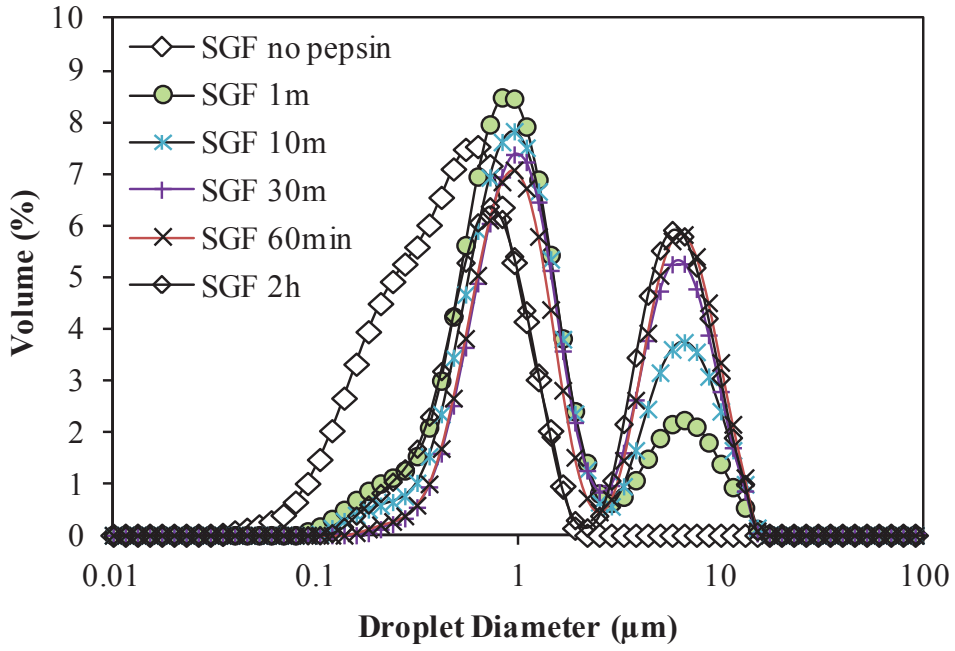


Figure 4.2 Particle size distributions of sodium caseinate-stabilized emulsions (20 wt% soy bean oil, 1.0 wt% protein) in SGF containing no pepsin and after digestion in SGF containing pepsin for 1, 10, 30, 60 min and 2 hrs.

The particle size distribution of emulsions studied using static light scattering (Malvern MasterSizer 2000) showed that the initial emulsion had a monomodal particle size distribution and did not change significantly when it was mixed with SGF without added pepsin (Figure 4.2). However, as shown in Figure 4.2, on mixing with SGF containing pepsin, the emulsion immediately became bimodal, with the second peak in the region of 2–15 μm and a corresponding decrease in the area of the first peak.

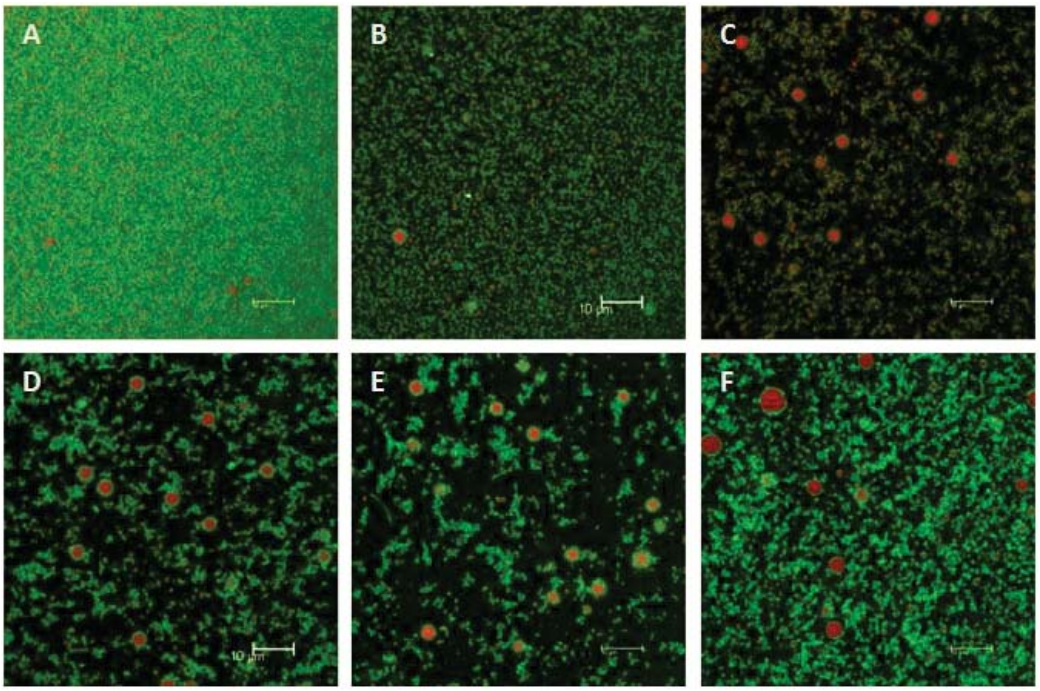


Figure 4.3 Confocal micrographs of sodium caseinate-stabilized emulsions: (A) original emulsion; (B) emulsion mixed with SGF (no pepsin); emulsions digested in SGF containing pepsin for (C) 10 min, (D) 30 min, (E) 60 min and (F) 120 min. All samples were stained with Nile Red (for oil) and Fast Green (for protein). Scale bar = 10 μm.

An increase in the area of the second peak in response to a relative decrease in the area of the first peak was observed with an increase in the digestion time. This indicated that flocculation and coalescence of the emulsion droplets occurred within the system by the action of pepsin.

The sodium-caseinate-stabilized emulsion and the emulsion mixed with SGF containing no pepsin (pH 1.6) had fine uniformly dispersed emulsion droplets (Figures 4.3A and 4.3B). However, clusters of aggregates were observed after mixing with SGF containing pepsin for 10 min (Figure. 4.3C). The aggregate size increased within 30 min of digestion and there were some relatively large droplets in the emulsion samples that

were digested for 30 min. This indicated that the presence of pepsin caused both flocculation and coalescence of the emulsion droplets.

4.3.1.2 Zeta potential

The zeta (ζ) potential of emulsions can be one of the important parameters that determine the stability of emulsions. In protein-based emulsions, the emulsion stability is generally driven by electrostatic repulsion and/or steric stabilization. Freshly prepared emulsions at pH 7 stabilized with sodium caseinate was anionic with an average zeta potential of -51.8 ± 1.00 mV. It was highly negative because the pH was appreciably above the pI for caseins (pH 4.6) (Mun et al., 2007). On mixing the emulsion with SGF without added pepsin at pH 1.6, the emulsion transformed into a cationic form with the zeta potentials of +19.8 mV because the pH is lower than pI for caseins (Sarkar et al., 2009b). In SGF containing pepsin, the zeta potential was +27.9 mV after 1 min and then remained constant during further digestion. A previous study reported by Sarkar et al. (2009) showed that in SGF containing pepsin, proteolytic action on the adsorbed protein from β-lg stabilized O/W emulsions caused a gradual loss of positive charge at the droplet surface (Sarkar et al., 2009b). However, this was not observed in this study, despite the fact that caseins adsorbed at the interfaces were hydrolysed into small peptides on mixing with SGF containing pepsin (Figure 4.5).

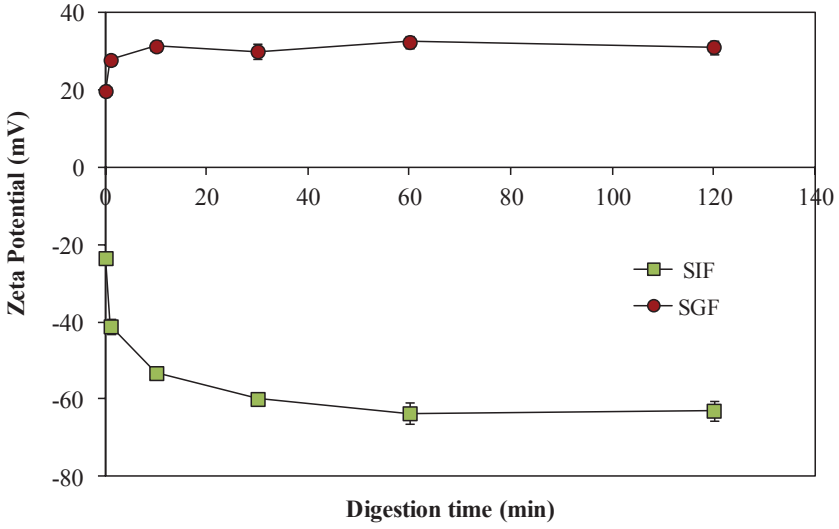


Figure 4.4 Changes in zeta potentials of sodium caseinate-stabilized emulsions during digestion in SGF (pH 1.6, pepsin) and SIF (pH 7.5, pancreatin and bile salt) as a function of digestion time. 0 min represents the emulsions mixed with SGF or SIF without containing enzymes (pepsin or pancreatin).

4.3.1.3 SDS-PAGE

Sodium caseinate proteins are comprised of four main types of caseins, such as α_{s1}-, α_{s2}-, β- and κ- caseins (Guo et al., 1995). These proteins have been reported to be susceptible to peptic hydrolysis during *in vitro* gastric digestion due to their relatively open, flexible conformational structures in their native state (de Wit, 1998, Guo et al., 1995, Dalgalarrondo et al., 1995) . SDS-PAGE was used to determine the hydrolysis of proteins adsorbed at the interface or present in the serum phase of the emulsions during gastric digestion (Figures 4.5A and 4.5B).

With an increase in the digestion time, the adsorbed caseins were rapidly hydrolysed to produce small peptides (Figure 4.5A). Within the first 1 min of digestion, the intensity of β-casein band decreased and the other three protein bands corresponding to α_{s1}-, α_{s2}- and κ- caseins all disappeared with the appearance of peptides at small molecular weights. After 10 min, all casein bands disappeared with only the peptides. This means that no intact casein remained on the interface after digestion for 10 min, and the surfaces of the emulsion droplets were probably covered by the small peptides. These results suggested the surface hydrolysis by pepsin could diminish the protective action of protein and remove the steric repulsion barriers, resulting in droplet aggregation and coalescence (Singh et al., 2009). Sarkar et al. (2009a) also reported that the hydrolysis of adsorbed β-lg from β-lg stabilized O/W emulsions by pepsin in SGF reduced the thickness of the adsorbed layer (Sarkar et al., 2009b). The electrostatic repulsive and steric barriers of the peptides remaining at the interface were not sufficiently strong to overcome the attractive forces between droplets. Therefore, extensive flocculation followed by coalescence of the droplets occurred. The presence of salts in the gastric juices also promotes droplet flocculation due to electrostatic screening effects (Singh et al., 2009, Sarkar et al., 2009b, Armand et al., 1999). Because of their flexible random coil structure, caseins in their native state are very susceptible to proteolysis by pepsin.

In the serum phase of emulsions, a protein band corresponding to α_{s2}-casein was not detected in all emulsion samples, including the original emulsion (Figure 4.5B, lane 3). This indicated that almost all α_{s2}-caseins were adsorbed onto oil droplets unlike a substantial amount of the other caseins (α_{s1}-, β- and κ-caseins) was present in the serum phase without being adsorbed at the interface. These unadsorbed caseins were also hydrolysed into small peptides rapidly after 1 min of digestion in SGF containing pepsin.

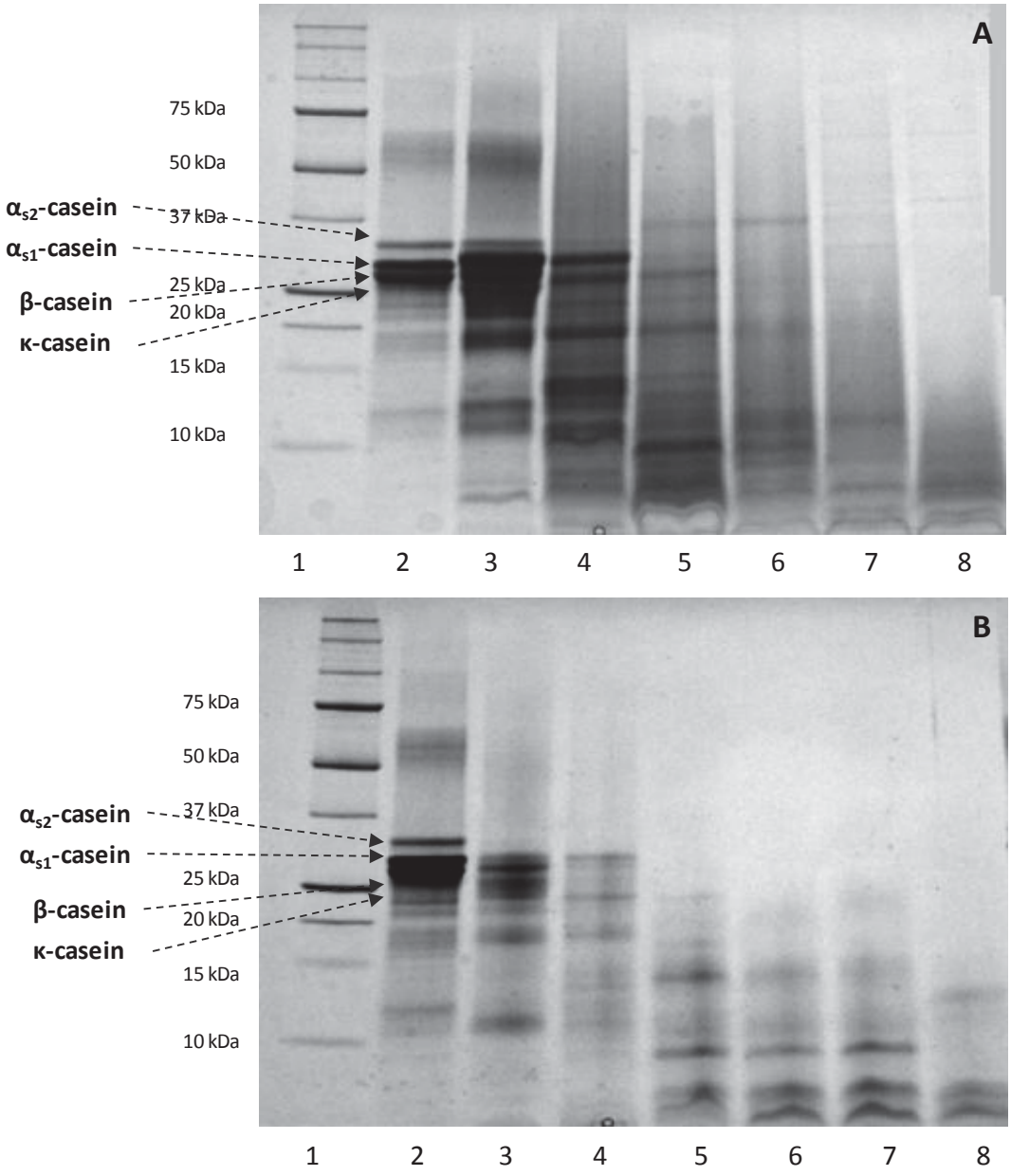


Figure 4.5 SDS-PAGE patterns of (A) the interfacial proteins obtained from the cream phase and (B) the proteins from the serum phase of sodium-caseinate-stabilized emulsions after digestion in SGF containing pepsin for different times: lane 1, molecule weight size markers; lane 2, sodium caseinate solution; lane 3, original emulsion; lane 4, digestion for 1 min; lane 5, digestion for 10 min; lane 6, digestion for 30 min; lane 7, digestion for 60 min; lane 8, digestion for 120 min.

4.3.2 Digestion of emulsions in SIF

4.3.2.1 Droplet size and microstructure

When this fresh emulsion was mixed with SIF without added pancreatin, the emulsion had no changes in the droplet size (data not shown). The addition of pancreatin together with bile salts into the sodium caseinate emulsion-SIF mixture resulted in changes in the particle size over time during digestion. There was a small change in $d_{3,2}$, but an appreciable increase in $d_{4,3}$ after 1 hr (Figure 4.6), indicating an occurrence of some big droplets. This was in agreement with the previous study (Mun et al., 2007). The significant increase in the particle size after 1 hr was probably due to the rupture of adsorbed casein layer at the oil-water interface, leading to the formation of large droplets, because caseins are highly susceptible to pancreatin hydrolysis (Guo et al., 1995). The significant increase in $d_{4,3}$ whereas no pronounced change in $d_{3,2}$ was observed could be because the volume mean diameter ($d_{4,3}$) is more sensitive to the presence of some large particles than does the surface mean diameter ($d_{3,2}$) (Mun et al., 2007, McClements, 2005).

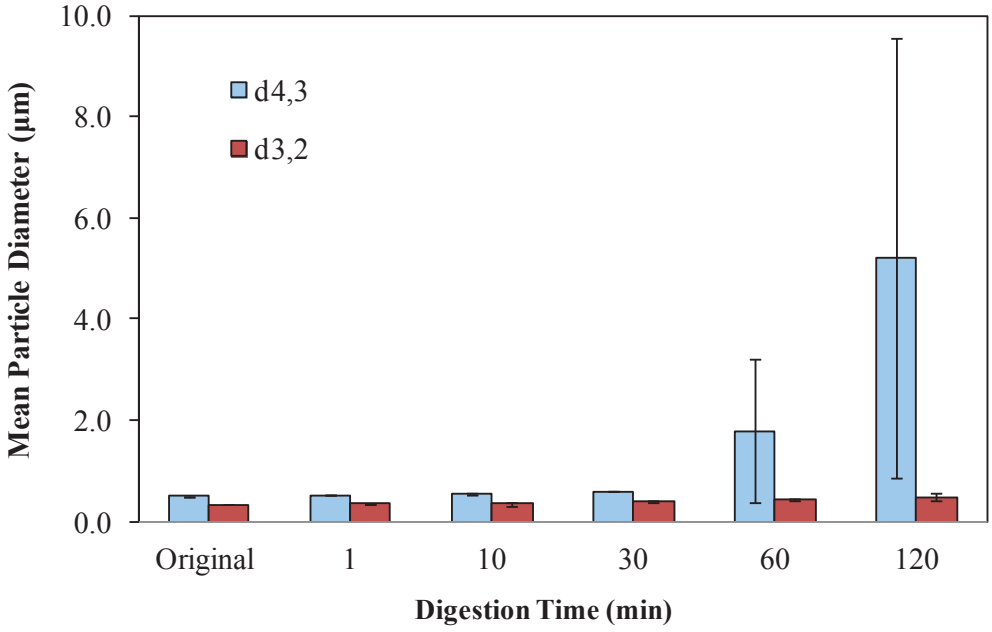


Figure 4.6 Changes in the particle diameters $d_{3,2}$ and $d_{4,3}$ of sodium caseinate-stabilized emulsions (20 wt% soy oil and 1.0 wt% NaCas) after digestion in SIF (pH 7.5, pancreatin and bile salts) for different times.

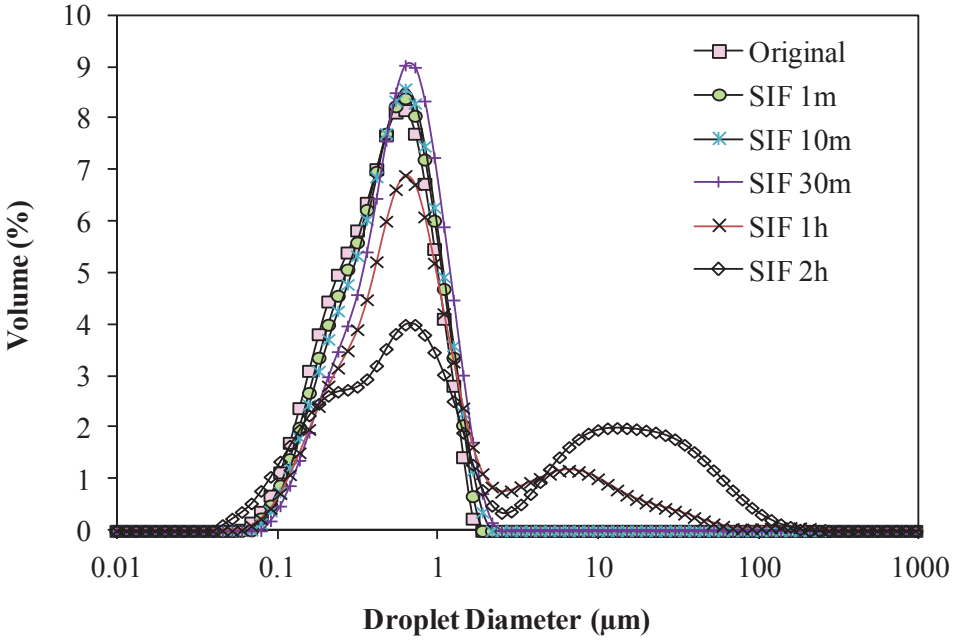


Figure 4.7 Changes in the particle size distributions of sodium caseinate-stabilize emulsions (20 wt% soybean oil and 1.0 wt% protein) after digestion in SIF (pH 7.5, pancreatin and bile salts) for different times.

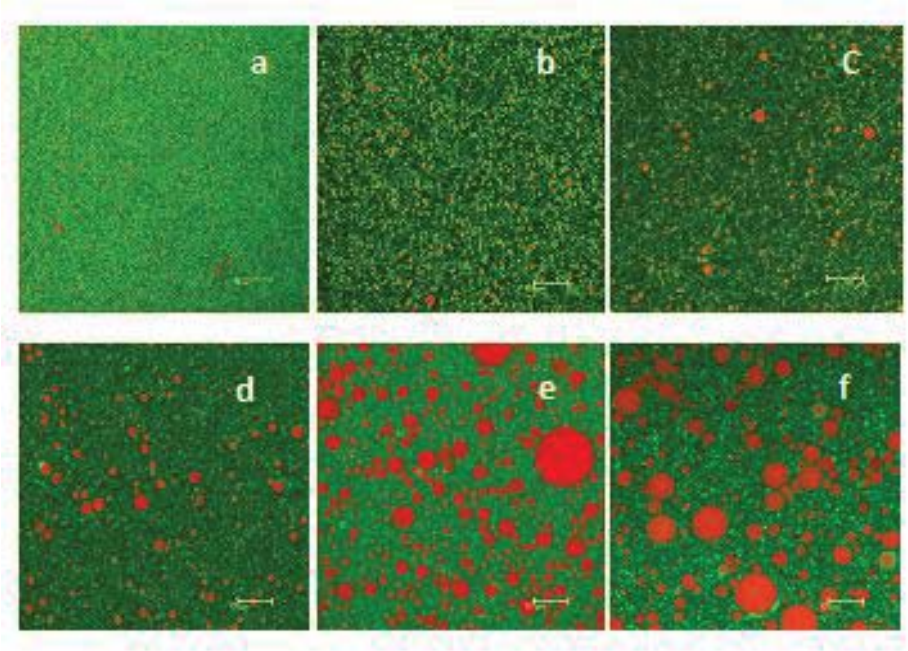


Figure 4.8 Confocal laser scanning microscope (CLSM) images of sodium caseinate-stabilized emulsions after digestion in SIF for different times. Original emulsion (a), and emulsions digested in SIF containing pancreatin and bile salts for 1 min (b), 10 min (c), 30 min (d), 60 min (e) and 120 min (f). Scale bar = 10 μm.

The particle size distributions of emulsions, shown in Figure 4.7, also illustrate that a substantial change in the size of emulsions occurred, especially after 1 hr of digestion, and that a pronounced change did not occur during digestion for short times in SIF. After 1 hr of digestion, the size distribution changed from monomodal with a peak at around 0.5 μm to bimodal with a second peak in the larger size region. The confocal microscopic analysis also confirmed the size distribution results, and the presence of large droplets was detected after 1 hr of digestion (Figure 4.8).

4.3.2.2 Zeta potential

As described above, the original sodium caseinate-stabilized emulsion at pH 7 had the zeta potential of -51.8 mV. When this emulsion was mixed with SIF (pH 7.5) containing bile salts without pancreatin, the zeta potential changed to -23.4 ± 0.47 mV (Figure 4.4). The decrease in the zeta potential might be due to an increased ionic strength resulting from the added salts (e.g. Ca^{2+} and Na^{+}) contained in SIF that could bind to the negatively charged amino acids of caseins adsorbed at the droplet interface.

When the emulsion was mixed with SIF containing pancreatin and digested for 2 hrs, there was a drastic increase in the zeta potential to -60 mV within 30 min and then its value remained relatively constant during further digestion. It has been shown that pancreatin could have displaced some caseins from the interface and hydrolysed the oil droplets, resulting in the formation and release of free fatty acids. Therefore, the zeta potential could be increased in its negative charge due to some hydrolyzed fatty acids retained at the interface (Sarkar et al., 2010b). In other words, the possible reasons for the increased negative zeta potential at the beginning of digestion for 30 min could be initiated by the displacement of caseins from the interfacial layer by bile salts or its direct adsorption on the interface that facilitates an access of pancreatin to the lipid core, thus the generation of negatively charged free fatty acids at the interface.

4.3.3 Sequential digestion of emulsions in SGF and SIF

4.3.3.1 Droplet size and zeta potential

After digestion in SGF for different times, the emulsion samples were then digested in SIF containing pancreatic lipase. The changes in the size of the emulsion droplets during digestion in SIF after digestion in SGF are shown in Figure 4.9. The average droplet size of emulsion samples added directly to SIF increased with an increase in the digestion time, mainly after 1 hr (Figure 4.6). The increase in size was more pronounced when the emulsion samples had been previously digested in SGF; the longer the digestion time in SGF, the larger was the droplet size for the same digestion time in SIF. For the emulsion samples digested in SGF for 30 min and 2 hrs, there was an significant increase from 1.61 to 16.16 μm and from 2.80 to 27.80 μm in $d_{4,3}$ after 2 hrs intestinal digestion (Figure 4.9).

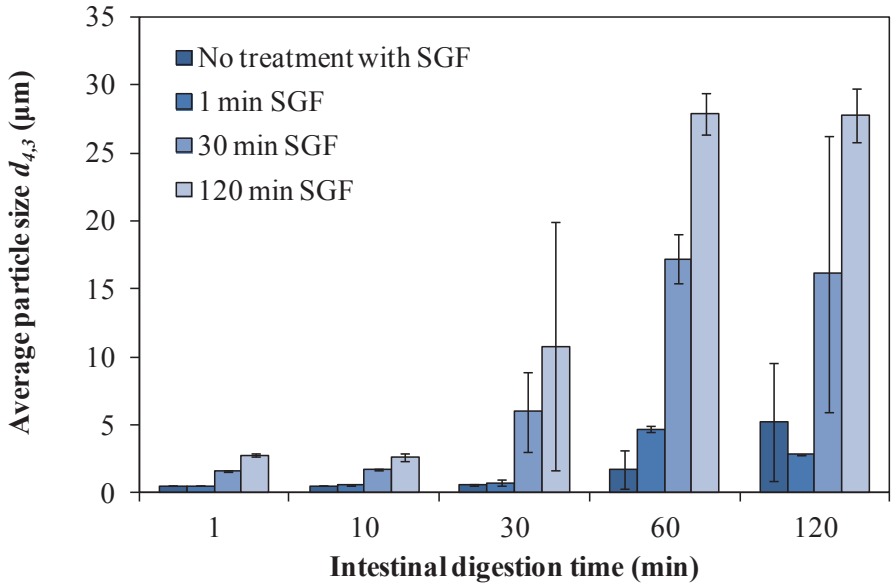


Figure 4.9 Change in average droplet size ($d_{4,3}$) of sodium caseinate-stabilized emulsions (20 wt% soya oil, 1.0 wt% protein) after digestion in SGF containing pepsin for 1, 30 and 120 min and then further digestion in SIF containing pancreatic lipase for different times.

The size distributions of emulsions during digestion in SIF after gastric digestion are shown in Fig. 4.10. As described above, the emulsion digested in SIF without digestion in SGF had almost no change in its size distribution with digestion in SIF for short times but the peak moved to a large size range after digestion for 1 hr (Figure 4.7). After digestion in SGF, the changes in the peaks during the subsequent digestion in SIF were

more rapid and the peaks were broader than the emulsion sample that was not digested in SGF (4. 10). After digestion in SGF for 30 min and 2 hrs, followed by digestion in SIF for more than 30 min, the size distribution became multimodal, with both a peak in the 0.1 μm range and a peak in the very large range (Figures 4.10A and 4.10B).

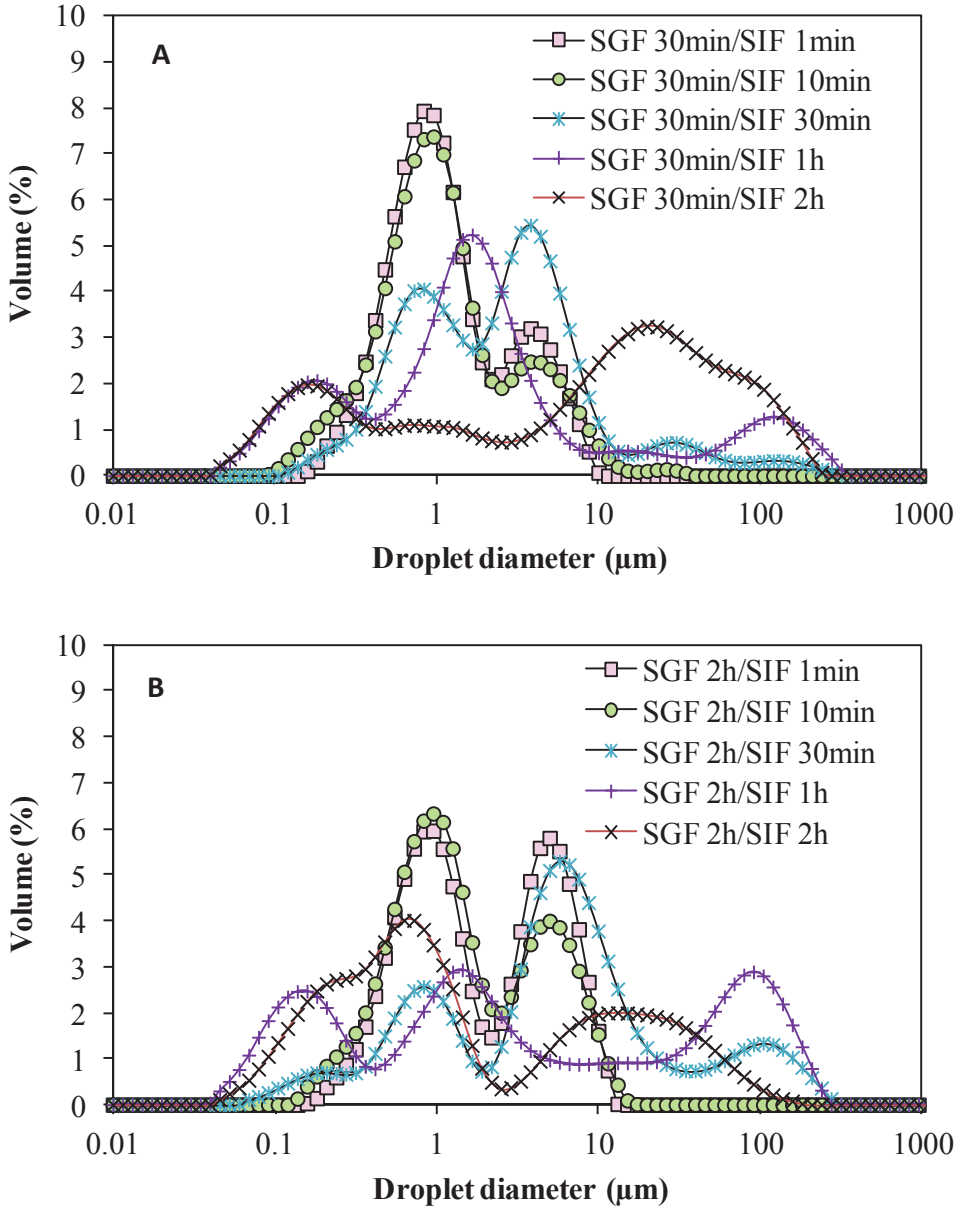


Figure 4.10 Particle size distributions of sodium caseinate-stabilized emulsions digested in SGF containing pepsin for (A) 30 min and (B) 2 hrs and then digested in SIF containing pancreatic lipase.

The zeta potentials of emulsions were measured during digestion in SIF after digestion in SGF for 30 min and 2 hrs (Figure 4.11). There was a more pronounced change in the droplet size due to the sequential digestion, it did not cause a significant difference. The

trend in changes in the levels of zeta potential with the digestion time during the subsequent intestinal digestion after gastric digestion was similar to that of the emulsions that was directly digested in SIF. As already described earlier, the sharp increase in the negative zeta potential within the first 30 min of digestion was believed to be due to the generation of free fatty acids hydrolyzed from the lipid core by pancreatin (Sarkar et al., 2009c).

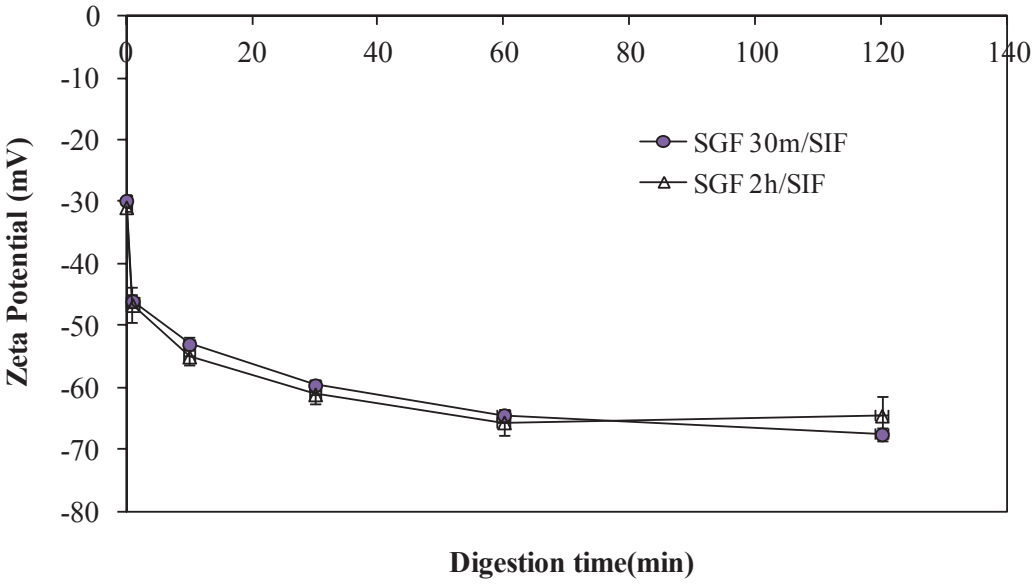


Figure 4.11 Zeta potentials of sodium caseinate-stabilized emulsions measured during digestion in SIF after digestion in SGF for 30 min and 2 hrs.

4.3.3.2 Microstructures of emulsions

Confocal micrographs of the emulsion samples digested in SIF after digestion in SGF are shown in Figure 4.12. The number of large emulsion droplets increased with increasing digestion time in all samples. There were some very large oil droplets in the samples that were digested for 2 hrs, especially in the samples that had previously been digested in SGF for 30 min or 2 hrs. This indicated that the increase in the size of the emulsion droplets during digestion in SIF was due to their coalescence. As well as the large oil droplets, there were many small particles in the emulsion samples that had been digested for a long time (2 hrs), which matched with the multimodal particle size distribution in those samples.

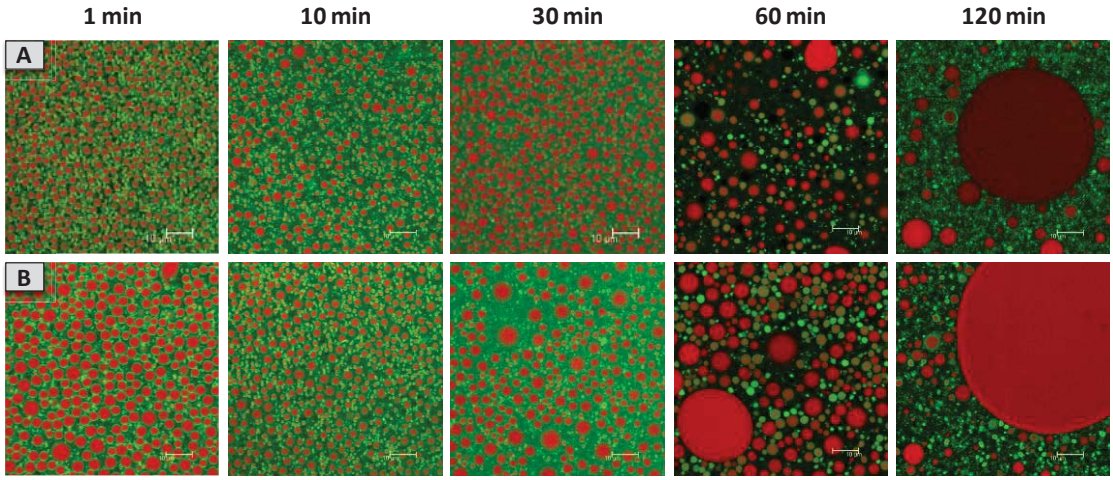


Figure 4.12 Confocal micrographs of sodium-caseinate-stabilized emulsions digested in SGF containing pepsin for (A) 30 min and (B) 2 hrs and then in SIF containing pancreatic lipase for different times. All samples were stained with Nile Red (for oil) and Fast Green (for protein). Scale bar = 10 μm.

In this work, the emulsion droplets underwent coalescence during digestion with pancreatic lipase in the presence of bile extract (Figures 4.10 and 4.12). This has been observed previously for protein-stabilized emulsions during in vitro digestion in simulated intestinal environments (McClements et al., 2008, Hur et al., 2011a). During digestion in SIF containing pancreatic lipase, the surface compositions of the droplets could change as interfacial proteins or peptides are further hydrolysed by serine proteases (trypsin and chymotrypsin) to produce even smaller peptides. In addition, more and more fatty acids and monoacylglycerols are released from the core of the droplets during lipid digestion. Fatty acids and monoacylglycerols are surface-active substances and accumulate at the surfaces of emulsion droplets (Staggers et al., 1990). These emulsion droplets with new surfaces consisting of small peptides, fatty acids and monoacylglycerols will be more susceptible to coalescence, because of their lower surface viscosity and elasticity. As a result, the original surface materials may not dominate the digestion behaviour of the emulsions at longer digestion times. The enhanced coalescence of the droplets during digestion in SIF for emulsion samples that had been pre-digested in SGF was probably caused by proteolysis of the interfacial protein by the pepsin-containing SGF. The surface consisting of small peptides was less efficient at preventing coalescence of the droplets in the SIF environment. However, molecular details of the sequential actions of pepsin and trypsin/chymotrypsin on

adsorbed proteins in emulsions are not known. Bile salts may also be involved in the process of destabilization, as these surface-active compounds displace the interfacial proteins/peptides from the surfaces of emulsion droplets.

A large proportion of the small particles generated at long digestion times (Fig. 4.12) can be attributed to the break-up of these large coalesced droplets. However, these small particles that were released from the large droplets may not have been similar to the original emulsion droplets. They were probably mixtures of lipolysis products, proteins, phospholipids and bile extract, in which the bile extract played the role of an amphiphilic agent. It is known that bile extract enhances the solubility of lipolysis products in the water phase through the formation of various vesicles that are soluble in water, thereby removing them from the site of emulsion droplets (Porter and Charman, 2001).The size and the structure of these vesicles are dependent on the concentrations of bile extract and components involved in the formation of the vesicles (Wiedmann and Kamel, 2002).

4.3.3.3 Free fatty acid release

The pancreatic-lipase-catalysed lipolysis of emulsion samples that had been previously digested in SGF containing pepsin was quantified by determining the amount of free fatty acids released under SIF conditions containing pancreatin and with and without added bile salts (Figure 4.13). There was no significant difference in the rate and the amount of free fatty acids released from the different emulsion samples, which indicated that the differences in the size and the surface layer of these emulsions after digestion in SGF containing pepsin had no apparent influence on the lipid digestibility. However, a significant difference was observed between the emulsion samples digested in the presence and absence of bile salts. In the presence of bile salts (Figure 4.13A), the release of free fatty acids increased up to 70 μmol after 2 hrs digestion. However, in the absence of bile salts (Figure 4.13B), it was much lower and there was a gradual increase in free fatty acids released to around 30 μmol after 2 hrs of incubation time. These results indicated that pancreatin was also able to bind to the interface of sodium caseinate-stabilized emulsions even in the absence of bile salts but its binding and hydrolysis activities of pancreatic lipase was enhanced by bile salts.

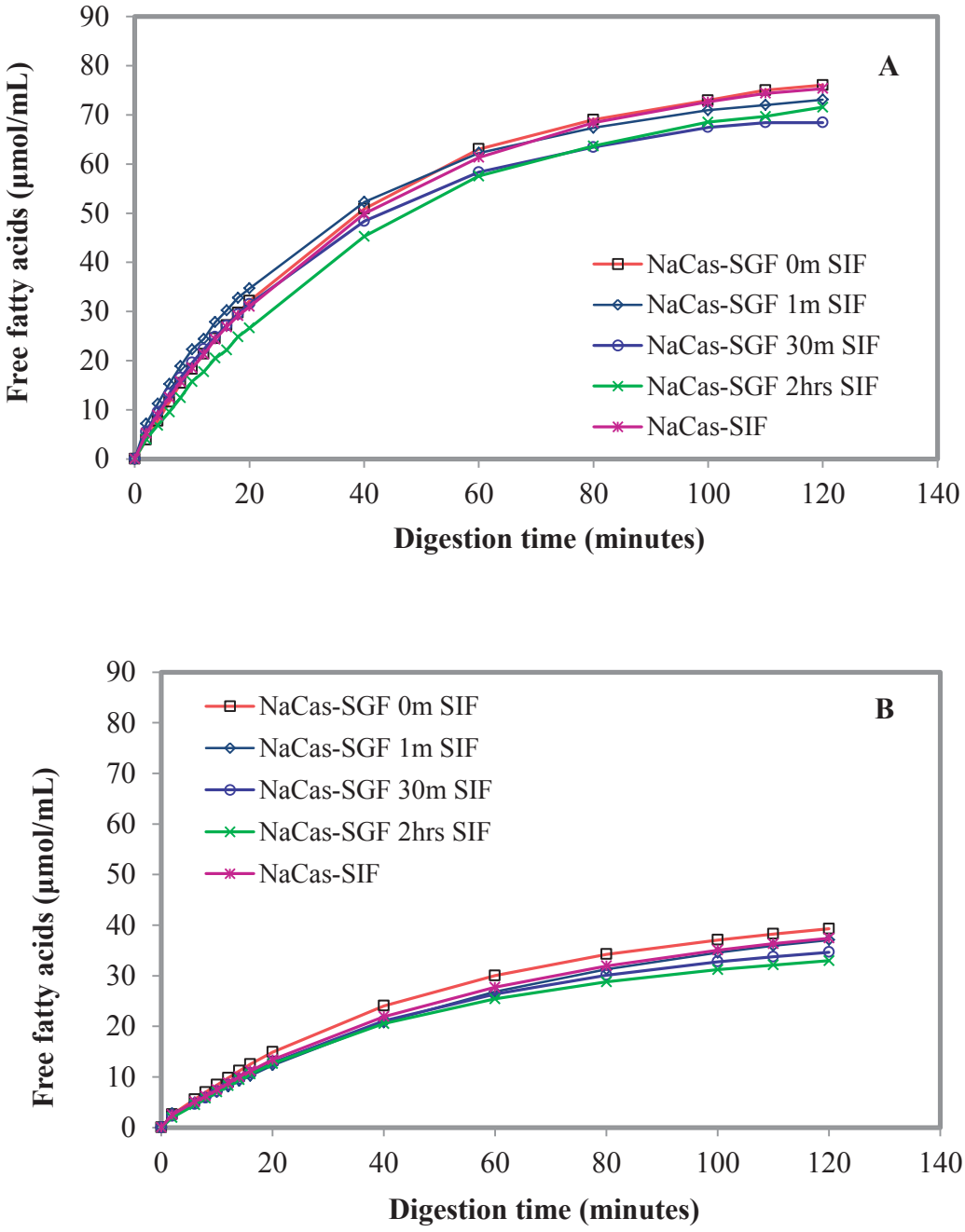


Figure 4.13 The release of free fatty acids (µmol/mL emulsion) hydrolyzed from NaCas-emulsions during digestion in SIF for 2 hrs after gastric treatment for different times. (A) SIF containing 5.0 mg/mL bile salts and 1.6 mg/mL pancreatin and (B) SIF containing 1.6 mg/mL pancreatin and no bile salts.

Some studies have shown the effect of the size, the surface layer and the structure of emulsion droplets on in vitro lipid digestion (Hur et al., 2011a, Wiedmann and Kamel, 2002, Porter and Charman, 2001). However, the initial surface layer of the emulsion droplets has only a limited impact on lipid digestion in the presence of bile salts (Hur et

al., 2009), probably because the bile salts, with their strong surface-active properties, displace interfacial material from the surfaces of emulsion droplets that are stabilized either by the initial surface protein layer or by proteolysis of those proteins. This appears to promote access of the active site of pancreatic lipase to the hydrophobic lipid core. This enhancement of the hydrolysis by pancreatic lipase eliminates the difference in the lipolysis caused by the changes in the size of the droplets and the proteolysis of the interfacial proteins (Singh and Sarkar, 2011, Pharmacopeia, 1995).

4.4 Conclusions

Sodium-caseinate-stabilized emulsions underwent extensive droplet flocculation, with some coalescence, after digestion in SGF, resulting in changes in the droplet size and the microstructure of the emulsions. These changes in physical properties can probably be attributed to proteolysis of interfacial proteins by pepsin under gastric conditions. In general, digestion in SGF containing pepsin accelerated the changes in the physical properties of the emulsion droplets during subsequent digestion in SIF containing pancreatic lipase. However, the changes in the size and the microstructure of the emulsions under gastric conditions did not appear to influence the rate and the extent of lipid digestion in the subsequent intestinal environment.

Chapter 5. In vitro Gastrointestinal Digestion of Oil-in-Water Emulsions Stabilized by Tween 20

5.1 Abstract

This study was to investigate the digestion behaviour of Tween 20-stabilized emulsions (1 wt% emulsifier and 20 wt% oil) that can occur during the *in-vitro* sequential digestion under simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) conditions. Changes in the particle size, zeta potential and microstructure of emulsions and the amount of fatty acids hydrolyzed from emulsified lipids were measured to characterize the emulsion digestion. Tween 20-stabilized emulsions did not show a significant change in their particle size and zeta potential during 2 hrs of digestion with SGF (pH 1.6, 34 mM NaCl, with or without 3.2 mg/mL pepsin). However, when the emulsions were digested with SIF (pH 7.5, 5.0 mg/mL bile salts, 150 mM NaCl, with or without 1.6 mg/mL pancreatin), the droplet size was increased considerably due to coalescence of droplets after 2 hrs of intestinal digestion. A gradual increase in the negative charge of zeta potential was also observed during the SIF digestion, changing from -14.7 ± 0.7 mV to -32.7 ± 1.5 mV. The sequential digestion of emulsions with SGF and SIF showed that the pattern of physicochemical changes occurring in emulsions was similar to that of emulsions digested with SIF only, except for the emulsion digested for 2 hrs in both SGF and SIF which exhibited some distinguished larger emulsion droplets. The results suggest that the pre-treatment with SGF did not significantly affect the sequential intestinal digestion of emulsions stabilized by non-ionic small molecule surfactant of Tween 20.

5.2 Introduction

Emulsions are colloidal dispersion systems playing an important role in many processed foods. One of the most important functions of emulsions, particularly oil-in-water emulsion systems, is their ability to incorporate and disperse lipophilic components (e.g. edible oils and fats) as small particles into aqueous liquid or solid food matrices. Food emulsions are stabilized by various types of emulsifiers, including some proteins (sodium caseinate, whey proteins, soybean proteins) and polysaccharides (gum Arabic) and small molecule surfactants.

Recently, *in vitro* digestion of emulsions has been a subject of great interest among scientists. Many studies have been carried out to establish the mechanisms of emulsified lipid digestion and absorption in the gastrointestinal (GI) tract (Hur, Joo, Lim, Decker, & McClements, 2011; McClements, Decker, Park, & Weiss, 2009; Singh, Ye, & Horne, 2009). Among them, the behaviours of emulsions stabilized by a surfactant or a combination of a surfactant and others (e.g. proteins and other surfactants) during gastric or intestinal digestion have been investigated (Li & McClements, 2011; Mun, Decker, & McClements, 2007; van Aken, Bomhof, Zoet, Verbeek, & Oosterveld, 2011). Van Aken et al. (2011) examined the differences in *in-vitro* gastric digestion behaviours of homogenized milk and emulsions stabilized by Tween 80, whey protein or a combination of whey protein and caseinate, and found that the emulsions stabilized by Tween 80 had a relatively high stability against flocculation and coalescence of emulsion droplets compared with others. This was due to the chemical nature of Tween 80 being non-ionic.

Tween 80 is a non-ionic surfactant derived from polyethoxylated sorbitan and oleic acid, therefore, the emulsion droplets stabilized by Tween 80 are not sensitive to pH and do not flocculate on decreasing the pH (How et al., 2011, van Aken et al., 2011). Another study reported by Mun et al. (2007) on the *in vitro* intestinal digestion of Tween 20 or protein stabilized emulsions showed that the emulsion droplets stabilized by Tween 20 were the least accessible to pancreatic lipase as Tween 20 is more surface-active than lipase so that lipase cannot bind to the oil droplet surfaces, therefore, they were the most stable to flocculation and coalescence than sodium caseinate or WPI stabilized emulsions (Gargouri, Julien, Bois, Verger, & Sarda, 1983; Mun, et al., 2007). Tween 20, known as polysorbate 20, is also a non-ionic small molecule surfactant and is chemically a polyoxyethylene derivative of sorbitan monolaurate. The addition of small molecule surfactants to protein-stabilized oil-in-water emulsions during *in vitro* digestion was also reported to inhibit pancreatic lipase activities. It was explained that the inhibition effect is because surfactants either fully or partially displace pancreatin molecules from the oil-water interface, thereby reducing the direct contact between lipid core and enzyme (Li & McClements, 2011; McClements, Decker, & Park, 2009).

However, the behaviours of *in vitro* intestinal digestion of emulsified lipids stabilized with small molecule surfactants after gastric treatment have not been studied. Small

molecule surfactants, such as Tween 20, 40, 60 and 80, are widely utilized as an emulsifier or detergent in food, biotechnology and pharmaceutical applications. Their hydrophilic-lipophilic balance (HLB) values are in the range of 8-17, which makes it suitable for an oil-in-water emulsion (Hsu & Nacu, 2003).

The aim of this study was to investigate changes in the physicochemical properties and lipid digestion of Tween 20-stabilized oil-in-water emulsions during digestion with simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and SGF/SIF (e.g. sequential digestion). The particle size, zeta potential and microstructures were measured to determine the digestibility of Tween 20-stabilized emulsions. The rate of free fatty acids hydrolyzed from emulsified lipids was also determined by titration in the absence or presence of bile salts during the sequential digestion with SGF and SIF.

5.3 Results and Discussion

5.3.1 In vitro SGF digestion of Tween 20-stabilzied emulsions

5.3.1.1 Particle size and size distribution

Oil-in-water emulsions (1 wt% Tween 20, 20 wt% soy oil, pH 6.71) stabilized by Tween 20 were prepared to investigate *in vitro* digestion behaviour under the conditions that simulate the GI tract. Initially, the mean particle diameter of Tween-20 stabilized emulsions was analysed, prior to its treatment with SGF containing pepsin, using a static light scattering technique. The surface ($d_{3,2}$) and volume ($d_{4,3}$) mean diameters of emulsion droplets were 0.233 ± 0.0021 μm and 0.401 ± 0.0042 μm, respectively (Fig. 5.1). Upon mixing with SGF (pH 1.6), the particle sizes of $d_{3,2}$ and $d_{4,3}$ were increased slightly to 0.281 ± 0.007 μm and 0.454 ± 0.0021 μm, respectively, and then remained relatively constant with marginal changes over time. After 2 hr incubation, the particle size appeared to be 0.261 ± 0.0007 μm and 0.428 ± 0.0 μm, respectively. It was also determined that the incubation of emulsions in SGF without the addition of pepsin showed a similar pattern with no significant change over time (data not shown). These results suggest that the enzyme pepsin has no significant influence on the digestion behaviours of emulsions stabilized by Tween 20.

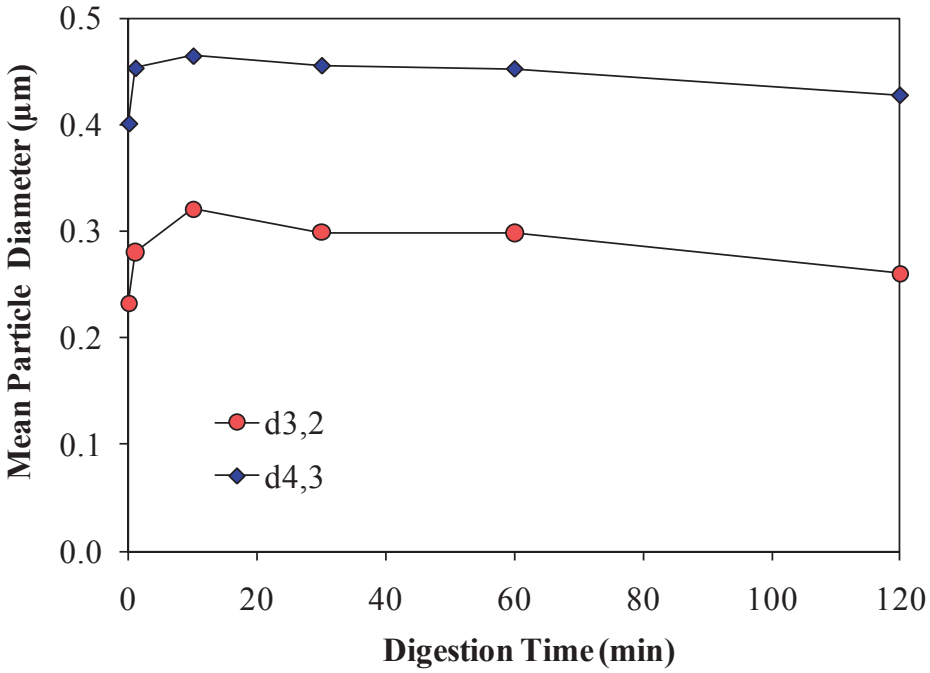


Figure 5. 1 The volume ($d_{4,3}$) and surface ($d_{3,2}$) mean particle diameters of Tween 20-stabilized emulsions (20 wt% soy oil and 1.0 wt% Tween 20) during digestion in SGF (pH 1.6) containing pepsin.

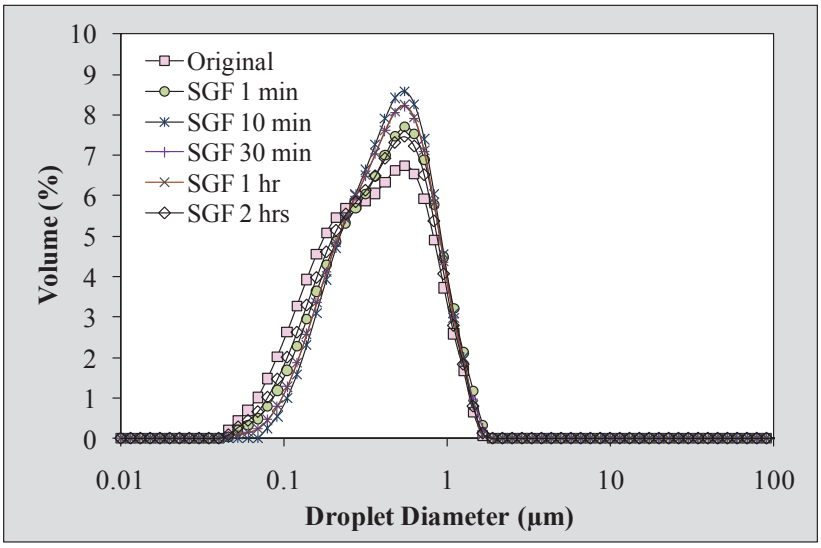


Figure 5.2 Particle size distributions of Tween 20-stabilized emulsions (20 wt% soy oil, 1.0 wt% Tween 20) before and duing digestion in SGF for 2 hrs.

Figure 5.2 shows the patterns of particle size distributions of original emulsion and emulsions treated with SGF containing pepsin for different periods of time. All emulsions were monomodal with a slight difference in their peak height and span. In

contrast to the slight increase in the mean particle size of emulsions when treated with SGF, the particle size distributions of these emulsions became slightly narrower with decreasing population of smaller particles as compared to the original control emulsion. The relative span values of their particle size distributions were in the range of 1.647-1.895 while the control emulsion had a span value of 2.083. Overall, Tween-20 emulsions were found to be fairly stable to acidic pH and resistant to hydrolysis by gastric enzyme pepsin because of the chemical nature of Tween 20 as emulsifier that is a nonionic, non-protein surfactant with no charge groups (Tatiana Dimitrova & Leal-Calderon, 1999; Dimitrova & Leal-Calderon, 2000; van Aken, et al., 2011). This means that the pH change has no significant influence on the emulsion stability and the enzyme, pepsin as proteinase, cannot hydrolyse the molecules of Tween 20 adsorbed on the surface of oil droplets (van Aken, et al., 2011). A study reported by Marciani et al. (2009) also showed that emulsions stabilized by Tween 60, which is also a non-ionic surfactant with similar characteristics to that of Tween 20, were stable under acidic conditions.

5.3.1.2 Zeta (ζ) potential

The electrical charge (i.e. zeta potential) on oil droplets in Tween 20-stabilized emulsions was also measured before and after the gastric digestion (Figure 5.3). The zeta potential of original emulsions stabilized by Tween 20 at pH 6.7 was -34.9 mV. This was rather larger than expected because as mentioned earlier Tween 20 is a non-ionic surfactant so it should be zero charge. Nevertheless, research studies have shown the negative zeta potentials of emulsions or nanodispersions stabilized by Tween 20, for examples, -23.93 mV for nanostructured lipid particles (How et al., 2011) and -15 to -19 mV for nanodispersions containing beta-carotene (Jean and Tan, 2010). Some other studies have also found that emulsions stabilized by Tween 20 are negatively charged (e.g. around -12 to -15 mV) at neutral pH (Hur et al 2009; Mun et al., 2007, Vladisavljevic and McClements 2010). It has been suggested that the negative electrical charge on oil droplets stabilized by Tween 20 could be due to the preferential binding of OH^- ions over H_3O^+ ions from water to the oil-water interface and/or the presence of anionic species (e.g. free fatty acids) in oils and/or surfactants (McClements, 2005; Mun et al., 2007; Hur et al 2009; Vladisavljevic and McClements 2010). Since Tween 20 is an emulsifier containing fatty acid esters in their molecules so Tween 20 can be hydrolyzed to release the fatty acids under certain conditions (e.g. enzyme lipase) prior

to or after emulsification and if this has occurred, the zeta potential of the emulsion systems stabilized by Tween 20 can be negatively charged (Mun et al., 2007)

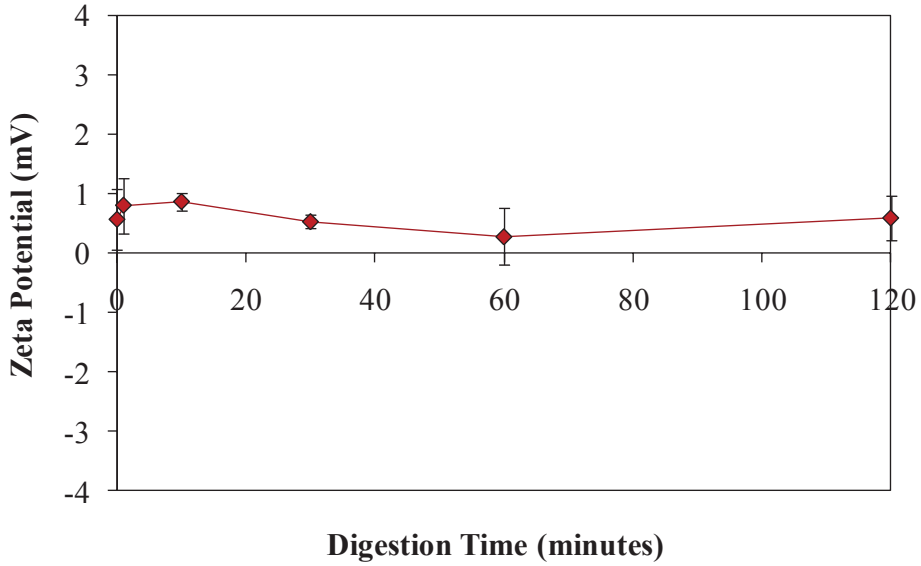


Figure 5.3 Electrical charges (ζ-potentials) of Tween 20-stabilized emulsions measured during digestion of emulsions in SGF (pH 1.6, containing pepsin) for 2 hrs.

When the original Tween 20-stabilized emulsion (pH 6.7) was mixed with SGF (pepsin and 34 mM NaCl) at pH 1.6, the zeta potential was decreased immediately upon mixing with SGF and changed from the negative charge of -34.9 mV to the positive charge of less than +1 mV. The zeta potential then remained constant thereafter till the end of 2 hrs incubation in SGF. Even though there was a significant reduction in the electrostatic repulsive force, the resulting emulsions still remained stable without exhibiting appreciable aggregation and coalescence of oil droplets. This can be inferred that the mechanism for the stability of emulsions by Tween 20 is due mainly to steric hindrance rather than electrostatic repulsion (Dimitrova & Leal-Calderon, 1999; McClements, 2005).

5.3.1.3 Microstructures of emulsions treated with SGF

As mentioned above, there was only a marginal change in the size distributions of Tween 20-stabilized emulsions during gastric digestion over 2 hrs in the presence of pepsin. The results could be verified by examining the microstructures of emulsions using a confocal laser scanning microscopy (CLSM) (Figure 5.4). The oil droplets of original Tween 20-stabilized emulsion were uniformly distributed (Figure 5.4a), and no appreciable changes were detected over 2 hrs during digestion in SGF (Figures 5.4b-f).

This confirms the high stability of Tween 20 emulsions under acidic condition. These results were quite different from those of sodium caseinate or WPI-stabilized emulsions where considerable agglomeration and aggregation of emulsion droplets were observed to occur in the protein-based emulsion systems after 30 min of digestion in SGF, which are discussed in the other chapters, but this was not seen for the emulsion stabilized by Tween 20.

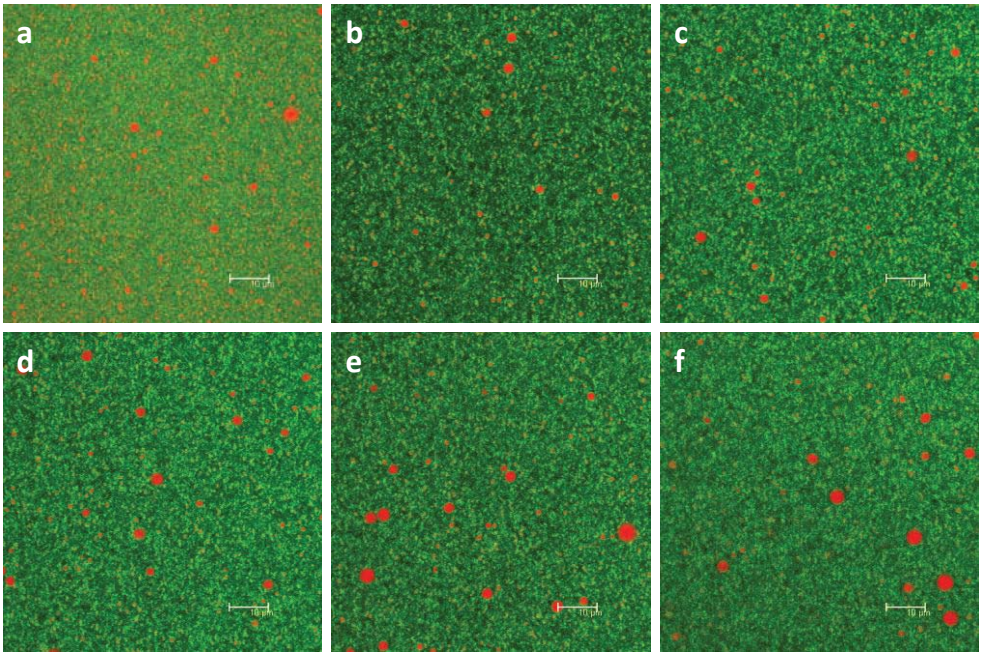


Figure 5.4 Confocal microscopic images of Tween 20-stabilized emulsions during digestion in SGF for 2 hrs. Original emulsion (pH 6.71) (a); emulsions in SGF digested for 1 min (b), 10 min (c), 30 min (d), 60 min (e) and 2 hrs (f). Scale bar represents 10 μm.

5.3.2 In-vitro SIF digestion of Tween 20-stabilzied emulsions

5.3.2.1 Particle size and size distribution

As previously mentioned, the freshly prepared Tween 20-stabilized emulsions had the volume mean ($d_{4,3}$) and surface mean ($d_{3,2}$) diameters of around 0.401 μm and 0.233 μm, respectively. When the original Tween 20 emulsions were mixed with SIF containing bile salts and pancreatin at pH 7.5 and incubated for 2 hrs, there was almost no change in the average droplet diameter ($d_{3,2}$) over time during 2 hrs incubation except a slight increase to 0.361 μm after 2 hrs (Figure 5.5). In case of the volume mean ($d_{4,3}$) diameter, it was found that the size remained constant as around 0.430 μm for the first 1 hr while it increased appreciably up to 2.249 μm after incubation for 2 hrs. The different results between $d_{3,2}$ and $d_{4,3}$ was because $d_{4,3}$ is more sensitive to big droplets than $d_{3,2}$

(McClements, 2005; Saehun Mun, et al., 2007), indicating that there were some larger droplets appeared in the system. This was able to be confirmed by the particle size distribution which differed greatly from the other samples (Figure 5.6). The particle size distribution was relatively uniform with a peak at around 0.54 μm within the first 1 hr of digestion while after 2 hrs of digestion, the particle size distribution became bimodal with a slight increase in the first peak shifting from 0.54 to 0.63 μm and an appearance of a small second peak in the region of 4.3-8 μm. These results indicated aggregation and coalescence of oil droplets and confirmed the aforementioned results of an increase in the volume mean diameter observed in this sample. The increase in droplet size after 2 hrs of digestion may be due to the displacement of Tween 20 by bile salts, providing an easy access of pancreatic lipase to lipid core and resulting in hydrolysis of lipid triglycerides by pancreatic lipase. Another possible mechanism for this could be due to partial hydrolysis of Tween 20 molecules by lipase from the interface, thus disrupting the interfacial layer and leading to aggregation and coalescence of oil droplets (Mun et al., 2007).

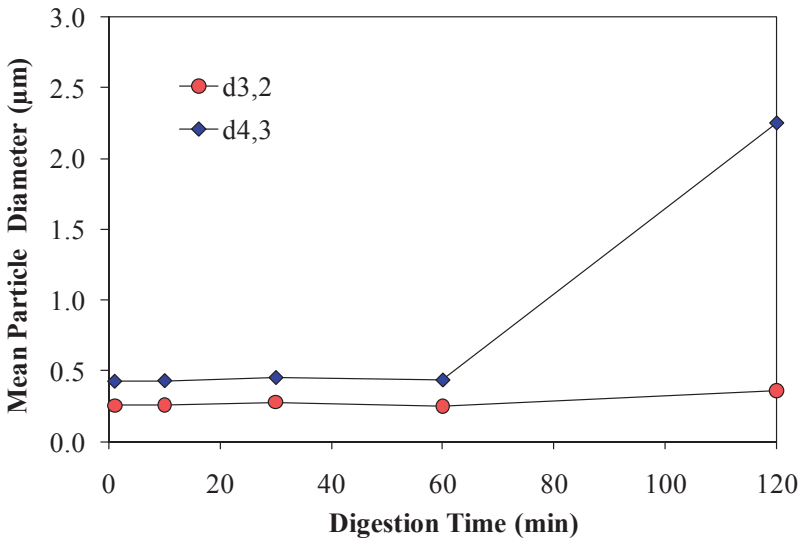


Figure 5.5 Changes in particle diameters $d_{3,2}$ and $d_{4,3}$ (μm) of Tween 20 emulsion droplets (20 wt% soy oil and 1.0 wt% Tween 20) during digestion in SIF (pH7.5, pancreatin and bile salts) as a function of time.

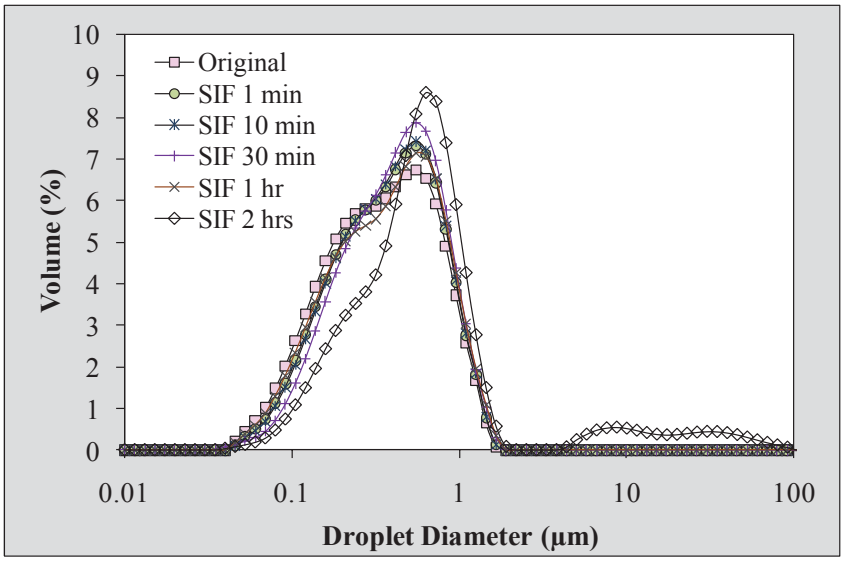


Figure 5.6 Changes in the particle diameters $d_{3,2}$ and $d_{4,3}$ (μm) of Tween 20 emulsions (20 wt% soy oil and 1.0 wt% Tween 20) during digestion in SIF (pH 7.5, pancreatin and bile salts) as a function of time.

5.3.2.2 Zeta potential

Upon mixing with SIF (5.0 mg/mL bile salts, 39 mM K_2HPO_4, 150 mM NaCl, 30 mM $CaCl_2$), the zeta potential on oil droplets of the original emulsions decreased immediately from -34.9 mV to -12.5 ± 0.72 mV. The possible reason for the decreased zeta potential could be due to the screening effect for the negative charges by cations (Na^+, Ca_2^{++} and K^+) added in SIF (Agboola & Dalgleish, 1995; Hur, et al., 2011; Kulmyrzaev, Sivestre, & McClements, 2000). Interestingly, the subsequent digestion over 2 hrs after mixing with SIF (with the presence of pancreatin) however resulted in a gradual increase in the zeta potential up to -32.7 ± 1.5 mV after 2 hrs (Figure 5.7). Although it is not certain, the possible explanation could be that anionic bile salts displaced Tween 20 molecules from the interface, promoting the accessibility of pancreatin to hydrolyse the lipid core (Gargouri, et al., 1983; Mun, et al., 2007). Moreover, pancreatin molecules might have displaced, interpenetrated and/or adsorbed onto the oil-water interface, resulting in a more negative zeta potential (Mun, et al., 2007).

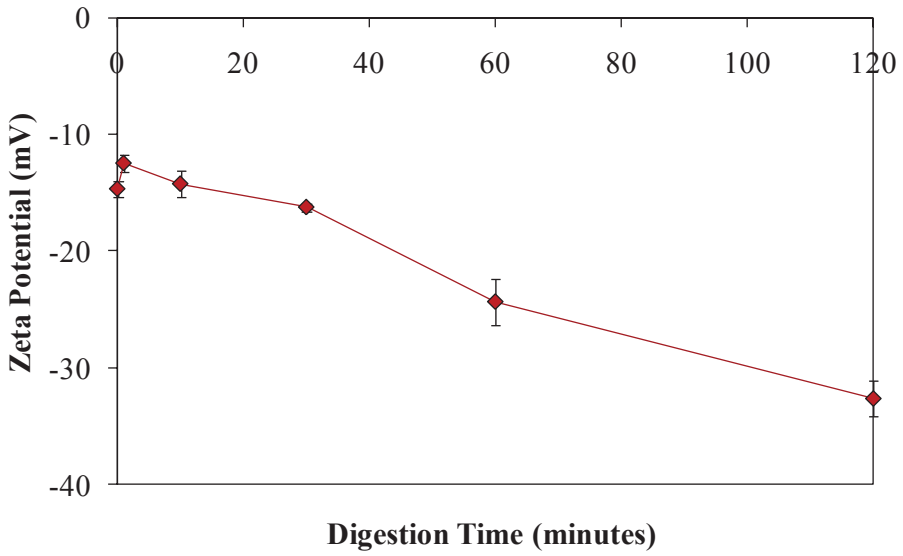


Figure 5.7 Zeta potentials of Tween 20-stabilized emulsions during digestion in SIF (pH 7.5, pancreatin and bile salt) as a function of incubation time.

5.3.2.3 Microstructures of emulsions treated with SIF

Confocal microscopic images (Figure 5.8) showed that there were no pronounced differences in the microstructures between the emulsion samples digested with SIF over time between 0 and 1 hr, indicating that the emulsions were stable within the first 1 hr of intestinal digestion. However, after digestion for 2 hrs, some bigger droplets due to coalescence were observed, which might be attributed to displacement of Tween 20 adsorbed on the surface of oil droplets by bile salts or other generated lipolytic products (e.g. mono- and diglycerides and free fatty acids) (van Aken, et al., 2011). The previous studies have reported that bile salts have an important role in lipid digestion by activating pancreatic lipase (Gargouri et al., 1983, Mun et al., 2007, Surh et al., 2006). The previous studies have reported that bile salts have an important role in emulsified lipid digestion by activating pancreatic lipase. For example, bile salts adsorb on the surfaces of emulsion droplets coated with lecithin by displacement of some lecithin, subsequently enabling the adsorption of pancreatic lipase to the surfaces coated with either lecithin and bile extract mixture. In this way, bile salts facilitate pancreatic lipase activity, allowing easy access of pancreatic lipase to the lipid core (Surh, Decker, & McClements, 2006).

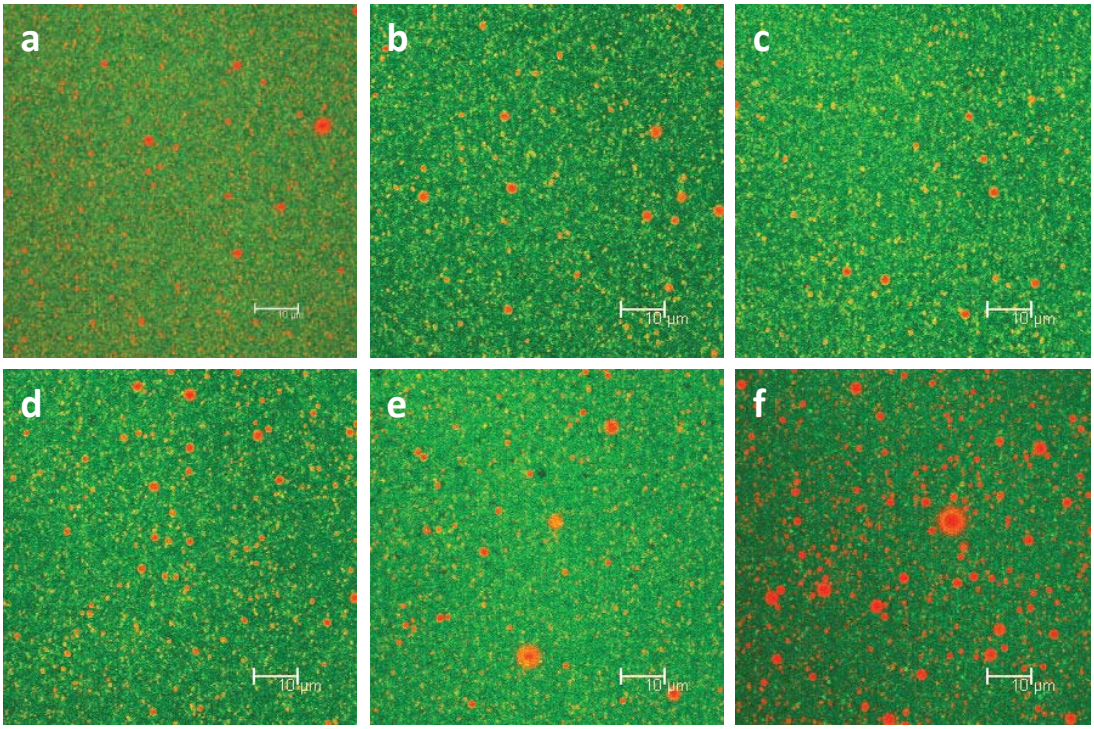


Figure 5.8 Confocal laser scanning microscope (CLSM) image of Tween 20-stabilized emulsions during digestion in SIF for 2 hrs. Original Tween 20-stabilized emulsion (a), emulsions digested in SIF containing pancreatin and bile salts for 1 min (b), 10 min (c), 30 min (d), 60 min (e) and 2 hrs (f). The scale bar represents 10 μm.

5.3.3 In vitro sequential digestion of Tween 20 emulsions in SGF and SIF

5.3.3.1 Particle size and size distribution

The sequential digestion of emulsions stabilized by Tween 20 with SGF and SIF was studied by first incubating emulsions with SGF at a 1:1 ratio for 30 min or 2 hrs and then treating the emulsion/SGF mixture with SIF at a 1:1 ratio for different periods of time (1, 10, 30 and 60 min and 2 hrs). Also, in order to determine the effect of gastric fluid on the subsequent intestinal digestion, the emulsion was mixed for 5 min with SGF without containing pepsin and then treated with SIF under the same conditions. The results are shown in Figures 5.9 and 5.10. For the emulsions treated first with SGF in the absence of pepsin, the mean droplet size remained relatively constant without a pronounced change during the subsequent digestion with SIF for the first 1 hr while after 2 hrs the droplet size $d_{4,3}$ increased from 0.461 to 0.810 μm. As a result of 2 hr sdigestion, the droplet size distribution became wider with its peak shifted to a larger particle size reigion. The similar trend was observed for the Tween 20 emulsion digested first with SGF for 30 min and then digested with SIF for 2 hrs. However, when the gastric treatment of Tween 20 emulsion was extended up to 2 hrs prior to its

intestinal treatment, the subsequent intestinal digestion caused a considerable change in the particle size and size distributions. The mean particle diameter ($d_{4,3}$) increased from 0.433 μm to 31.8 μm after 2 hrs. This resulted in a multimodal size distribution and a shift of the size distribution to a much larger size region with a corresponding decrease in the number of small droplets and an increase in larger droplets, suggesting the significant coalescence of emulsion droplets occurred in the system. This was confirmed by the microstructural examination of the corresponding emulsions as shown in Figure 5.11. In addition, the emulsions digested with SIF for shorter time than 30 min or 1 hr also exhibited some noticeable changes when treated with SGF for 2 hrs prior to the intestinal treatment. Overall, the results indicated that significant changes in the droplet size and size distribution occurred when the SIF digestion time applied was 2 hrs, regardless of the prior SGF digestion time. This was similar to the results shown in Figures 5.6 and 5.8 for the emulsion digested directly with SIF for 2 hrs without the gastric treatment. These results suggested that the gastric treatment prior to the intestinal digestion had an impact on the subsequent intestinal digestion only when the emulsion was pre-treated with SGF for a certain period of time (e.g. 2 hrs).

5.3.3.2 Microstructures of Tween 20 emulsions

The microstructures of Tween 20-stabilized emulsions were examined using CLSM after the sequential digestion with SGF and SIF in the presence and absence of pepsin (Figure 5.11). The microscopic images showed that irrespective of the SGF digestion time, all emulsions remained stable against flocculation and coalescence during the first 30 min of subsequent intestinal digestion while a number of larger droplets appeared in all emulsions after 2 hrs of the intestinal digestion. Also, some very large droplets appeared in the emulsions that had been treated with SGF for 2 hrs and then SIF for 2 hrs. The examination of microstructures of these emulsions confirmed that the results of significant increases and changes in the particle size and size distribution of emulsions described earlier were due to the presence of these larger droplets. The results imply that the gastric treatment for a longer time does affect the physicochemial changes of Tween 20 emulsions during the subsequent intestinal digestion. This result was different from our expectation because Tween 20 is a non-ionic surfactant so pepsin as proteinase cannot hydrolyse Tween 20 adsorbed on the surface of droplets and the interface is believed to be well protected against hydrolysis during the gastric digestion (Saehun

Mun, et al., 2007; van Aken, et al., 2011). This means that the gastric treatment prior to the intestinal digestion should not induce a significant influence on Tween 20 emulsions. However, as already described above, the subsequent intestinal digestion after gastric treatment for 2 hrs resulted in a significant droplet change, particularly after 2 hrs of intestinal digestion. The particle size distributions were bimodal with an increased second peak after 2hrs sequential digestion in SIF appeared in the region of 4-200 μm (Figure 5.10C) and the corresponding results were detectable from the microstructures of emulsions by CLSM (Figure 5.11C).The reason for this is not understood clearly. The treatment of emulsions with SGF for a shorter time (e.g. 30 min) also caused some changes but its extent was substantially less significant.

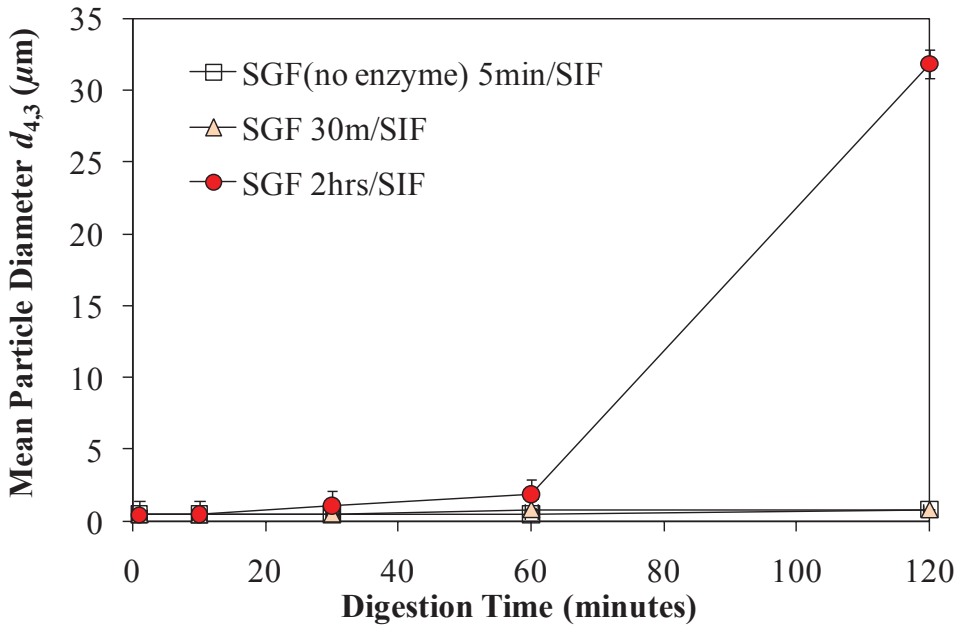


Figure 5.9 Mean particle diameters $d_{4,3}$ (μm) of Tween 20-stabilized emulsions during digestion in SIF (containing pancreatin) over 2 hrs after gastric treatment as a function of incubation time.

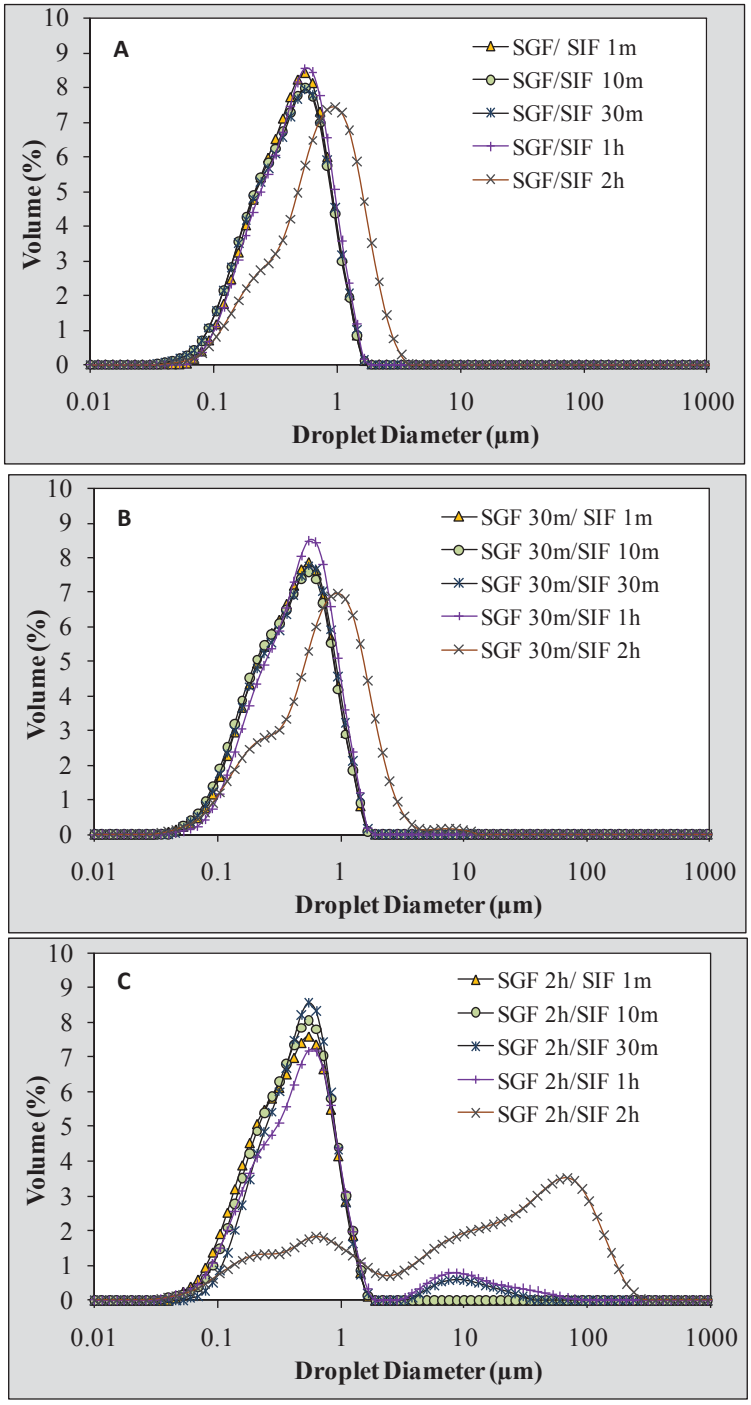


Figure 5.10 Particle size distributions of Tween 20-stabilized emulsions measured during sequential digestion with SGF and SIF. (A) after mixing with SGF (without pepsin stirring for 5 minutes); (B) and (C) after mixing with SGF (containing pepsin) for 30 and 120 min, respectively.

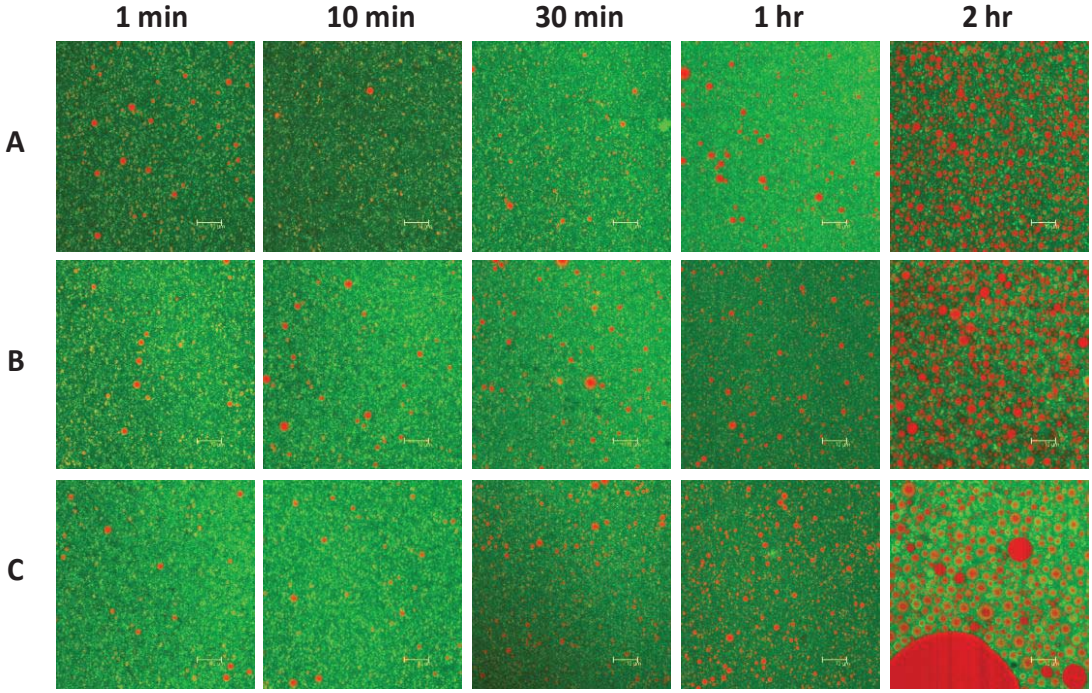


Figure 5.11 Representative confocal laser scanning microscopic (CLSM) images of microstructures of Tween 20-stabilized emulsions taken during sequential digestion in SIF for different times after gastric treatment. (A): emulsions with pre-treatment with SGF containing no pepsin; (B) and (C): emulsions with pre-treatment with SGF containing pepsin for 30 min and 2 hrs, respectively. The scale bar represents 10 μm.

5.3.3.3. Zeta potential

As described above, Tween 20-stabilized emulsions mixed with SGF (pH 1.6) had a small positive charge below + 1 mV and its zeta potential remained constant without a noticeable change over time during 2 hrs of gastric treatment. Although the zeta potential was close to 0, the emulsions maintained high stability against aggregation and coalescence of droplets which indicates that Tween 20 stabilized emulsions are quite resistant to destabilization at low pH. When the mixture of Tween 20 emulsion and SGF was further mixed with SIF (pH 7.5) for sequential digestion, the zeta potential changed from positive to negative and a gradual increase in the negative charge from -12.5 mV to -32.7 mV was observed over time during 2 hrs of the intestinal digestion period.

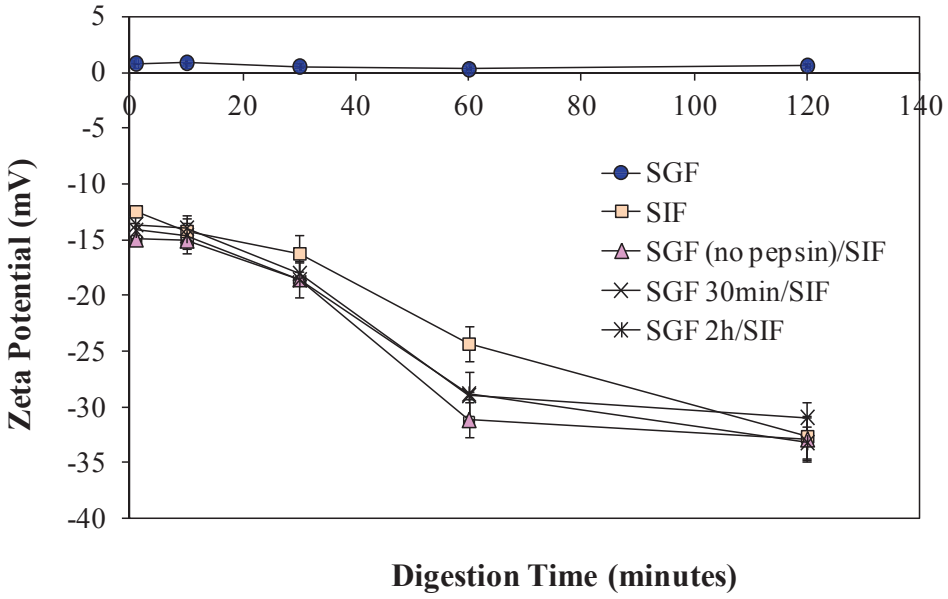


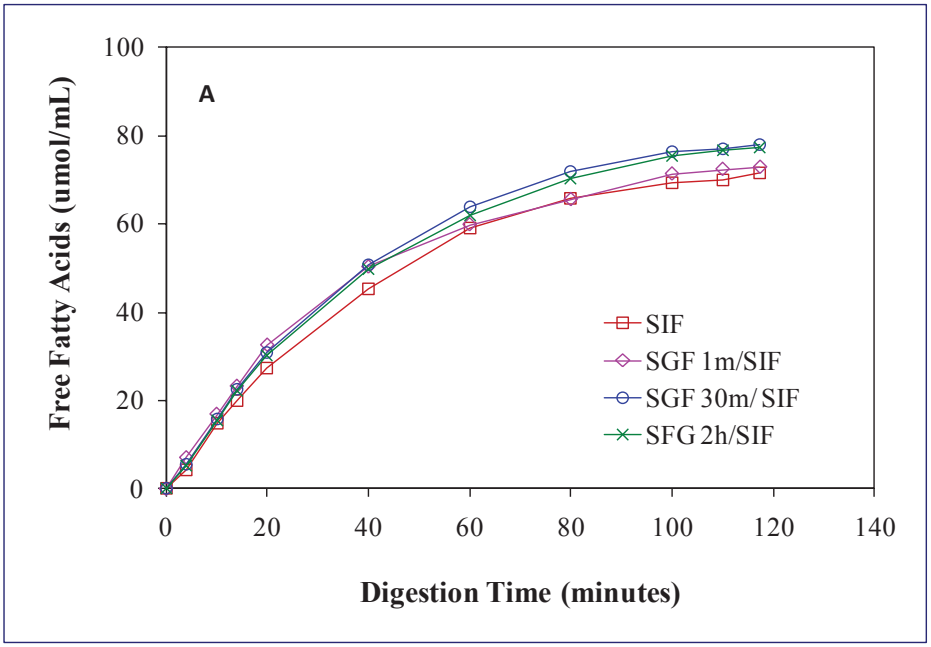
Figure 5.12 Zeta potentials of Tween 20-stabilized emulsions during digestion with SGF, SIF and SGF/SIF.

The zeta potential of emulsions is fairy dependent on pH and ionic strength, especially emulsion systems stabilized by ionic emulsifiers (proteins and carbohydrates) (Li & Tian, 2007; Mun, Decker, Park, Weiss, & McClements, 2006; Wickham, Garrood, Leney, Wilson, & Fillery-Travis, 1998). The possible reasons for the changes in the zeta potential of Tween-20 stabilized emulsions with non-ionic surfactant observed in this study were described in the above. During 2 hrs of the intestinal digestion after the gastric treatment at different time intervals (1 min, 30 min and 2 hrs), the trend of changing the zeta potentials was similar to that of Tween 20 emulsions digested in SIF only without the pre-treatment with SGF. The electrical charge tended to be slightly more negative during the intestinal digestion after gastric treatment (Figure 5.12). The increase in the negative charge could be due to both anionic bile salts and pancreatin that displaced and adsorbed onto Tween 20 molecules at the interface (Mun, et al., 2006; Wickham, et al., 1998). The data obtained were in agreement with the previous studies which also showed an increase in the negative charge of Tween 20-stabilized emulsion during the intestinal digestion (Gargouri, et al., 1983; Saehun Mun, et al., 2007). In this study, it was observed that the gastric treatment prior to the intestinal digestion did not significantly affect the zeta potentials of Tween 20 emulsions.

5.3.3.4. Hydrolysis of fatty acids

The hydrolysis rate of fatty acids from emulsified lipids stabilized by Tween 20 was determined over 2 hrs during the intestinal digestion of emulsions in the presence and absence of bile salt after their first treatment with SGF for different times (Figure 5.13). In the absence of bile salts, the amount of free fatty acids released after 2 hrs by pancreatic lipase was less than 50 μmol /mL (Figure 5.13B). There appeared to be a lag phase of about 20 min before any free fatty acids were produced, which was quite different from that of sodium caseinate and WPI-stabilized emulsions. This phenomenon was also observed by Mun et al. (2007). In their study, pancreatic lipase was found to access easily to the emulsified lipid core in emulsions stabilized by sodium caseinate, WPI or lecithin, but not in those stabilized by Tween 20 in the absence of bile salts. Their explanation for this was due to the ability of the highly surface active Tween 20 at the surface of oil droplets to prevent the enzyme from coming into close proximity to the emulsified lipids (Saehun Mun, et al., 2007).

The presence of the lag phase was probably attributed to the time taken for pancreatic lipase to displace Tween 20 molecules from the interface (Mun, et al., 2007). Based on this assumption, after 20 min, pancreatin gradually displaced or adsorbed onto some of Tween 20 molecules from the interface, causing lipolysis of emulsified lipids and generation of free fatty acids. After a certain time (around 110 minutes), the rate of free fatty acid released was relatively slow although triglyceride was not fully hydrolysed, which could be due to the inhibition of lipase activity by the adsorption of decomposed products (e.g. fatty acids) (Brockman, 2000; Mun, et al., 2007). In this study, the amount of free fatty acid released would be 454 μmol/mL if emulsified lipid triglycerides were fully hydrolysed (the calculation is attached in Appendix 3), indicating that about 11% of the total fatty acids were hydrolysed in the absence of bile salts.



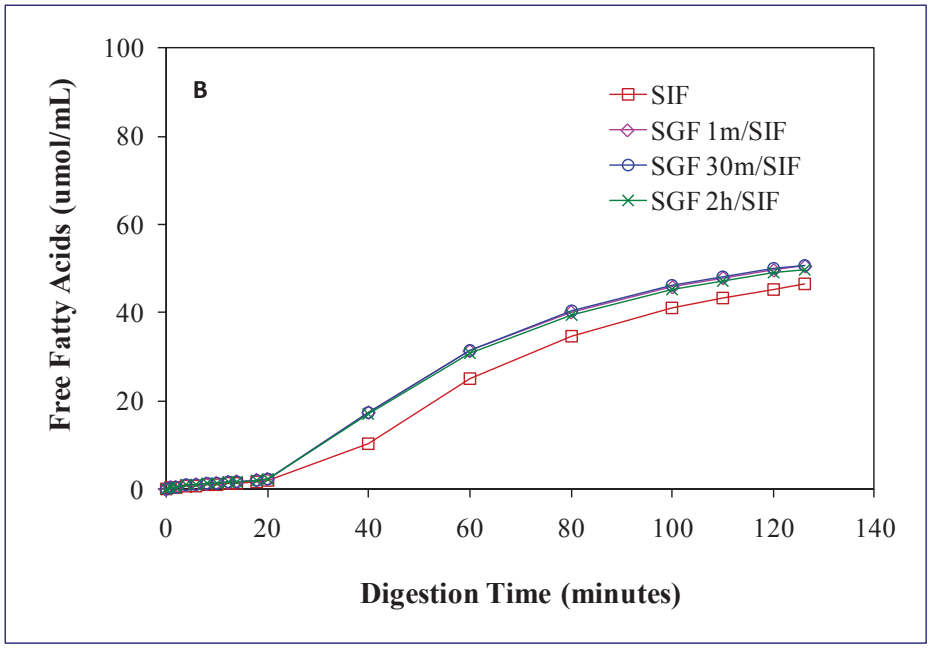


Figure 5.13 The release of free fatty acids (μmol/mL emulsion) hydrolyzed from Tween-20 emulsions during digestion in SIF for 2 hrs after gastric treatment for different times. (A) SIF containing 5.0 mg/mL bile salts and 1.6 mg/mL pancreatin and (B) SIF containing 1.6 mg/mL pancreatin and no bile salts.

In the presence of bile salts, the amount of free fatty acid released increased up to more than 70 μmol/mL emulsions (15% of fatty acids were hydrolysed) after 2 hrs of intestinal digestion of Tween 20 emulsion (Figure 5.13A), which was slightly higher than the case in the absence of bile salts. No lag phase was observed in the system. This could be due to displacement or adsorption of bile salts onto Tween 20 molecules upon

incubation, thereby facilitating lipase activity by providing an easy access to reach the oil droplet core. Previous researches suggested that the addition of bile salts to phosphatidylcholine-stabilized emulsions disrupted the phopholipids packing, leading to a formation of a mixed phospholipid/bile interface. This facilitated the pancreatic lipase activity as lipase further adsorbed to the surfaces of droplets coated with either lecithin and/or bile extract (Mun, et al., 2006; Wickham, et al., 1998). Similar to that of sodium caseinate and WPI-stabilized emulsions, the gastric treatment of Tween 20 emulsions before the intestinal digestion did not significantly influence the amount of free fatty acids released. There was little difference in the amount of free fatty acids released during the intestinal digestion of Tween 20 emulsion after their gastric treatment for different times.

5.4 Conclusions

Tween 20-stabilized emulsion showed only marginal changes during *in vitro* gastric digestion, which suggests that Tween 20 is fairly stable in the acidic conditions. When Tween 20 emulsions were mixed with SIF, the average droplet diameter $d_{4,3}$ and size distribution increased after 2 hrs digestion and an increase in the negative zeta potential was also observed along with the digestion time. The sequential digestion in both SGF and SIF showed similar results as in SIF, however, the emulsion droplets showed a more pronounced increase in $d_{4,3}$ during the intestinal digestion when the pre-gastric treatment used was for 2 hrs. Free fatty acid was generated due to lipid digestion by pancreatic lipase during intestinal digestion. In the absence of bile salts, a lag phase of 20 minutes was observed during intestinal digestion and the amount of free fatty acids hydrolysed was lower than 50 μmol/mL after 2 hrs intestinal digestion. In the presence of both bile salts and pancreatin, no lag phase was observed and the amount of free fatty acid released increased up to 70 μmol/mL emulsions. These results indicated that the gastric treatment did not significantly affect the physicochemical properties of emulsions stabilized by Tween 20 during their subsequent intestinal digestion. However, some changes in the average droplet size, size distribution, zeta potential and microstructure of Tween 20 emulsions were observed when the emulsions were digested in either SGF or SIF, especially for 2 hrs, regardless of the pre-gastric treatment.

Chapter 6. In Vitro Gastrointestinal Digestion of Oil-in-Water Emulsions Stabilized by WPI

6.1 Abstract

The digestion behaviour of whey protein isolates (WPI)-stabilised emulsions was examined under simulated intestinal fluid (SIF) conditions (pH 7.5, 2.5 mg/mL bile salts and 0.8 mg/mL pancreatin) after their first treatment with simulated gastric fluid (SGF) (pH 1.6 and 3.2 mg/mL pepsin). The physicochemical properties of emulsions, such as droplet size, zeta potential, microstructure, surface protein content and the amount of free fatty acids released, were measured. The results indicated that WPI emulsions underwent extensive droplet coalescence during SGF digestion followed by some degree of coalescence during the subsequent SIF digestion. When WPI emulsions were treated with SGF, α-LA and a portion of β-lg proteins adsorbed at the interface were hydrolyzed by pepsin, resulting in small peptides being produced as characterised by SDS-PAGE. As a result, the emulsion droplet sizes increased as a function of incubation time. After WPI emulsions were further treated with SIF after the gastric digestion containing pepsin, the zeta potentials increased drastically from -22.8 ± 1.0 mV to -49.1 ± 2.5 mV after 2 hrs. This could be attributed to the displacement of adsorbed β-lg by bile salts at the interface that allows easy access for pancreatin to the lipid core, leading to the generation of free fatty acids and the disruption and coalescence of emulsion droplets. The amount of free fatty acids released was determined by means of pH stat-titration. Bile salts were found to influence the release of free fatty acids significantly which was in agreement with the previous studies. In the absence of bile salts, the amount of free fatty acids released was about 48 μmol/mL emulsion after 2 hrs of intestinal digestion, whereas it increased significantly to 70 μmol/mL emulsion in the presence of bile salts. Moreover, the treatment with SGF did not affect the release rate of free fatty acids during the subsequent intestinal digestion. These results further confirm the theory that most of the lipid digestion is completed in small intestine.

6.2 Introduction

Dietary lipid play an important role in the human diet, providing energy, essential nutrients and bioactive components (Sandra et al., 2008). They may be consumed in different forms, such as bulk fats, fats trapped in solid matrices and emulsions (Mun et

al., 2006). It is critical to understand the digestion behaviour of dietary lipids ingested in the human body. Due to the complexity of *in vivo* test, *in vitro* digestion models have been largely applied to understand and characterise the digestibility and changes in food systems occurring during their passage through simulated mouth and gastrointestinal (GI) tract (Sarkar et al., 2009b, Sarkar et al., 2010b, Sarkar et al., 2009c, Mun et al., 2007, Beysseriat et al., 2006, Kaur et al., 2010a, Kaur et al., 2010b, Malaki Nik et al., 2010b, van Aken et al., 2011).

One of the important food systems is oil-in-water (O/W) emulsion as it has an important influence on the food microstructure and is also a common delivery system for water-insoluble lipid compounds in foods. It is important to understand the digestibility and stability of emulsions during transition through the GI tract in association with their microstructure and properties. Food emulsions usually experience a significant environmental change, for example, a significant decrease in pH when they enter the stomach with the acidic gastric fluid containing enzymes (pepsin and gastric lipase) and many electrolytes (Cl^-, Na^+, Ca^{2+}) (Singh et al., 2009, Dalgleish, 2003, McClements, 2005). When the emulsions further enter the intestinal tract, they encounter more complex physiological conditions, including a drastic pH increase, mixing with various enzymes (pancreatic lipases) and surface active agents (bile salts) and an exposure to different ionic strength and electrolytes (Singh et al., 2009, McClements et al., 2008). This implies that changes in the structure and properties of food emulsions occurring during their passage through the GI tract can be quite different, depending on their interfacial layer composition and structure, since their sensitivity to droplet aggregation and coalescence against environmental factors, such as pH and ionic strength, and their resistance to enzymatic degradation are affected significantly by the nature and types of emulsifiers (e.g. proteins, polysaccharides, and small molecule surfactants) used to have stabilized the emulsion droplets.

Whey proteins are defined as a fraction of proteins remaining soluble in milk after precipitation of caseins in milk at pH 4.6 (McClements, 2004, Dalgleish, 2004). The whey protein fraction represents about 20% of the total bovine milk protein and consists of mainly β-lactoglobulin (β-Lg) and α-lactoablumin (α-La), including some other minor whey proteins, such as bovine serum albumin (BSA), lactoferrin and

immunoglobulins (IgG). Some important biological functions of whey proteins are: β-lg for vitamin A transfer, α-LA for lactose synthesis, BSA for fatty acid transfer and IgG for passive immunity respectively (de Wit, 1998). These days, whey proteins are considered as one of the major important food proteins because of their multiple protein functionalities, such as gelling, emulsifying, and foaming abilities, and its nutritional value, especially for neonate (de Wit, 1998, Guo et al., 1995, Chatterton et al., 2006, Malaki Nik et al., 2010b). As emulsifiers, whey proteins are able to readily adsorb at the oil and water interface and form a dense layer at emulsion droplet surfaces that can provide the stability of emulsions to flocculation and coalescence (Chatterton et al., 2006, Malaki Nik et al., 2010b). They stabilize emulsions by a combination of electrostatic and steric repulsive forces (Agboola and Dalgleish, 1995).

In recent years, the *in vitro* gastric digestion of whey proteins-stabilized emulsions has been largely investigated (de Wit, 1998, Dalgalarrondo et al., 1995, Reddy et al., 1988, Malaki Nik et al., 2010b, Kim et al., 2007, Kitabatake and Kinekawa, 1998, Vermeirssen et al., 2005). β-lg in solution has been reported to be resistant to the acids and proteolytic enzymes present in the stomach (de Wit, 1998, Dalgalarrondo et al., 1995, Reddy et al., 1988, Malaki Nik et al., 2010b, Kim et al., 2007). Interestingly, β-lg as emulsifier adsorbed onto the surface of O/W emulsion droplets has been shown to be hydrolysed by pepsin during *in vitro* gastric digestion. The improved digestibility of β-lg when present at the interface is due to its unfolding and conformational rearrangement at the interface that reveal the peptic cleavage sites (Sarkar et al., 2009b). In contrast, α-La in solution can unfold and be readily digested by pepsin in the stomach at pH ≤4.0, with the subsequent generation of small peptides (Malaki Nik et al., 2010b, Kim et al., 2007, Papiz et al., 1986, de Wit, 1998). However, heat treatment could alter the susceptibility of whey proteins to pepsin. A study reported by Malaki et al. (2010b) on the *in vitro* gastric digestion of O/W emulsions stabilized by WPI showed that with pre-heat treatment of emulsion and solution up to 85^{o}C, β-lg was more susceptible to pepsin and hydrolysed into small peptides, while α-La was resistant to pepsin hydrolysis (Malaki Nik et al., 2010b).

The lipid digestion of O/W emulsions stabilized by whey proteins using *in vitro* intestinal digestion model has also been widely investigated, including the role of bile salts and pancreatic lipase activity (Sarkar et al., 2009c, Sandra et al., 2008, Mun et al.,

2007, Sarkar et al., 2010b). Sakar et al. (2009c) reported the effect of bile salts on β-Lg-stabilized O/W emulsions during intestinal digestion (containing bile salts and no enzyme). The zeta potential was observed to be more negative in the presence of bile salts due to competitive interfacial displacement of β-lg by bile salts. Another study by Sandra et al. (2008) on the effect of cross-linking protein on the *in vitro* intestinal digestion of emulsified corn oil by pancreatic lipase demonstrated that pancreatic lipase was able to adsorb to emulsion droplet surfaces and access to the emulsified lipids, regardless of the initial interfacial composition, during intestinal digestion. The presence of bile salts was reported to facilitate the pancreatic lipase adsorption onto the O/W emulsion interface. Mun et al. (2007) reported that the lipid digestion of three different O/W emulsion systems stabilized by sodium caseinate, WPI and Tween 20. It was found that a greater amount of fatty acids was released in the presence of both bile extract and pancreatic lipase than the pancreatinic lipase only, indicating that bile extract facilitated the emulsified lipid digestion.

There is scant limited information available about the sequential digestion of O/W emulsions stabilized by WPI in both SGF and SIF. The objective of this study was to investigate the understanding of digestion behaviour of WPI-stabilized emulsions when subjected to both SGF and SIF sequentially. Changes in the size, zeta potential and interface composition of WPI emulsions were determined. The amount of free fatty acids hydrolysed from WPI emulsions was measured.

6.3 Results and discussion

6.3.1 WPI-stabilized emulsion behaviour in SGF

6.3.1.1 Droplet size, zeta potential and microstructure

Changes in the size and properties of emulsions stabilized by WPI occurring during the GI digestion at 37^{o}C were determined using *in-vitro* model systems. Initially, freshly prepared WPI emulsions (1 wt% WPI and 20 wt% soybean oil) were measured for their pH and particle size. The pH was 7.2 and the mean particle diameters, $d_{3,2}$ and $d_{4,3}$, were 0.37 ± 0.042 μm and 0.68 ± 0.031 μm, respectively (Fig. 6.1). When this emulsion was mixed with the SGF (pH 1.6) containing pepsin and then incubated for 2 hrs, the particle size seemed to increase slightly over time but its increase was very minor (Fig. 6.1). After 2 hrs of incubation, the average droplet diameter ($d_{3,2}$ and $d_{4,3}$) increased to

0.42 ± 0.039 μm and 0.77 ± 0.050 μm, respectively. As a result of a very small change in the droplet size, the size distributions of WPI emulsions appeared to be the same as the original emulsion with no pronounced difference although there was a slight shift towards a little larger size range (Fig. 6.2). The microscopic images of WPI emulsions taken with a confocal laser scanning microscopy, shown in Figure 6.3, also indicated no appreciable changes in the microstructure of WPI-stabilised emulsions due to the gastric digestion. All these results indicate that WPI-stabilized emulsions were stable without droplet aggregation and coalescence under the gastric condition (pH 1.6) containing pepsin.

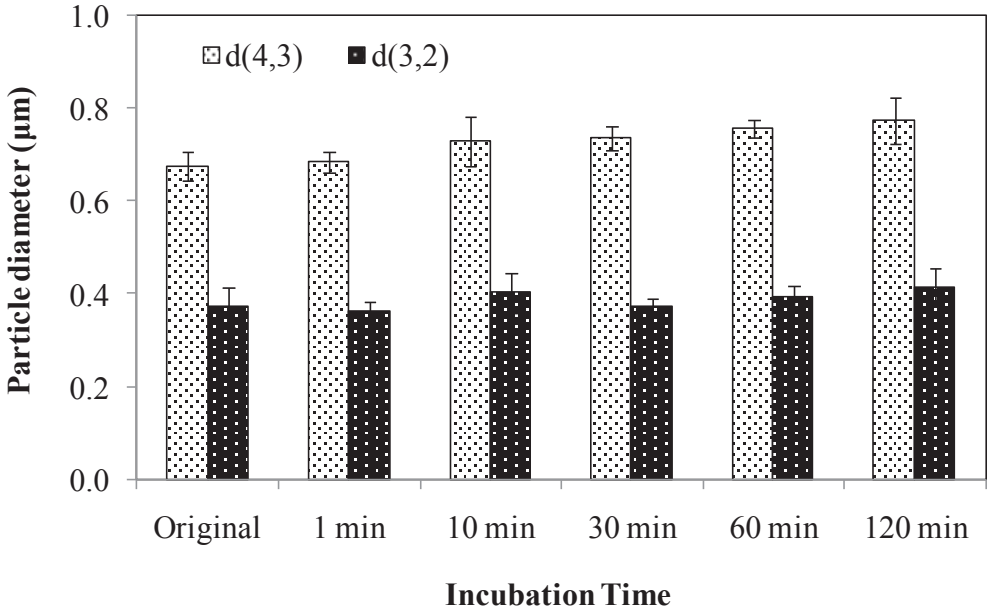


Figure 6.1 The volume ($d_{4,3}$) and surface ($d_{3,2}$) mean particle diameters of WPI-stabilized emulsions (20 wt% soy oil and 1.0 wt% WPI) during digestion in SGF (pH 1.6) containing pepsin.

A recent study by Malaki Nik et al. (2011) has shown a similar result of minor changes in WPI-stabilised emulsions upon incubation with SGF containing pepsin. They showed a slight increase in the particle size $d_{3,2}$ from 0.14 to 0.20 μm and the formation of a little wider size distribution. These authors described that WPI emulsions remained stable without droplet aggregation even though there was some hydrolysis of adsorbed whey proteins at the interface by pepsin. They suggested that the stability of emulsions against droplet aggregation and coalescence might have been maintained by the binding of proteins present in the serum phase to the interface and the attachment of peptides hydrolysed from adsorbed proteins to the interface.

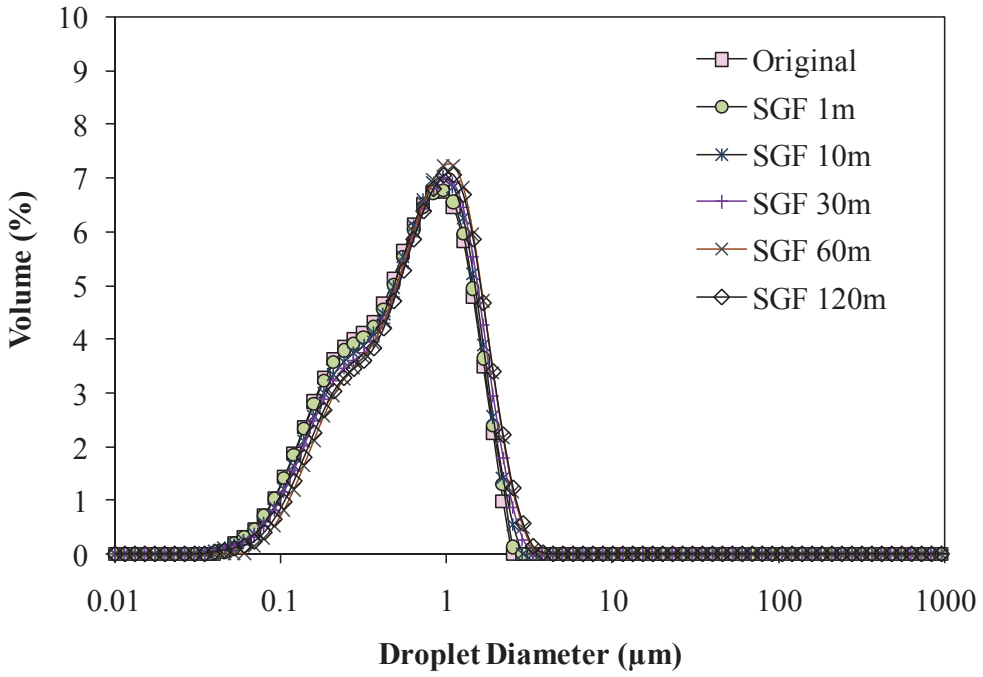


Figure 6.2 Particle size distributions of WPI-stabilized emulsions (20 wt% soy oil, 1.0 wt% WPI) before and during digestion in SGF for 2 hrs.

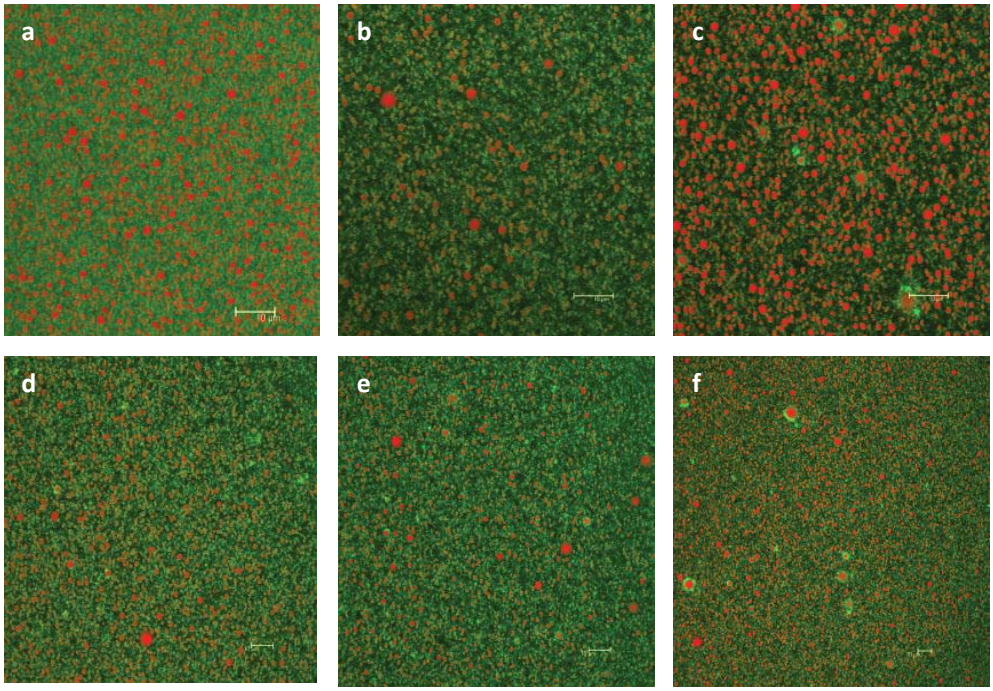


Figure 6.3 Confocal laser scanning microscope (CLSM) image of WPI-stabilized emulsions during digestion of emulsions in SGF (pH 1.6, containing pepsin) for 2 hrs. Original emulsions (a), original emulsions digested in SGF containing pepsin for 1 min (b), 10 min (c), 30 min (d), 60 min (e) and 2 hrs (f). The scale bar represents 10 µm.

The zeta (ζ)-potential of WPI emulsions was also measured to investigate changes in the interfacial layer properties of emulsion droplets stabilized by WPI during *in vitro* gastric

digestion. The original emulsion at pH 7.2 had the ζ-potential of -56.8 ± 2.3 mV. Upon mixing with SGF at pH 1.6, the ζ-potential changed from negative to positive with 32.3 ± 1.5 mV (Fig. 6.4), regardless of the presence and absence of pepsin in SGF. This value remained relatively constant over time during 2 hrs of the gastric digestion. This was also in agreement with the previous study of gastric digestion of WPI emulsions reported by Malaki Nik et al. (2010a, 2011). The change in the ζ-potential from the negative to positive values was due to a pH change to below the isoelectric point of whey proteins (p*I* ~5.2) (Sarkar et al., 2009b, Malaki Nik et al., 2010a, Hong and McClements, 2007). The ζ-potential of protein-based emulsions is positive at pH < p*I* while it changes to negative at pH > p*I*, which is due to changes in the electrical charge of amino acids comprising proteins according to the pH level (Hong and McClements, 2007).

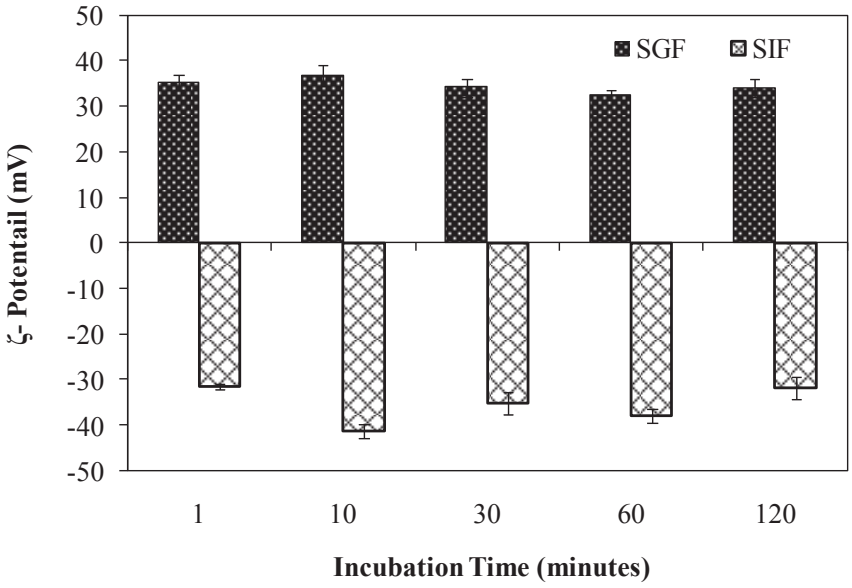


Figure 6.4 The ζ-potentials of WPI-stabilized emulsions measured during digestion in SGF (pH 1.6) and SIF (pH 7.5, pancreatin and bile salt), respectively, as a function of incubation time.

6.3.1.2 SDS-PAGE analysis

The hydrolysis of adsorbed whey proteins at the interface by pepsin during incubation of WPI-stabilized emulsions with SGF was studied by SDS-PAGE (Fig. 6.5). Analysis of SDS-PAGE gels showed that whey proteins adsorbed on the surface of emulsion droplets were mainly β-lg with a relatively small amount of α-La (Fig. 6.5A). The

amount of these two proteins observed to be different could be attributed to differences in their concentrations in the WPI powder used in the emulsion preparation and in their binding affinities to the interface of oil and water. A substantial amount of these two major whey proteins was also observed to be present in the aqueous (serum) phase without being adsorbed at the interface (Fig. 6.5B). In the serum phase, some other minor whey proteins corresponding to relatively high molecular weights, one of which is believed to be bovine serum albumin (BSA), were also detected in SDS-gels (Fig. 6.5B). These minor whey proteins were hydrolyzed upon the SGF digestion, and this phenomenon agreed with the previous study that BSA was reported to be sensitive to protease digestion (Zayas, 1997).

Before treatment with SGF containing pepsin, the samples of washed cream displayed two major bands of β-lg and α-La (Fig. 6.5A, lane 4) as already described above. Within the first 1 min of gastric digestion, the intensity of these two protein bands decreased with the appearance of faint bands of peptides with lower molecular weights (<10 kDa) that were diffused into the bottom of the gel, indicating the results of hydrolysis by pepsin. The intensity of these small molecular peptides from the washed cream phase appeared to decrease gradually over time during 2 hrs incubation, indicating their release from the interface into the serum phase. At the end of 10 min of incubation (Fig 6.5A, lane 6), α-La band was observed to disappear almost completely whereas an appreciable amount of β-lg still remained intact at the interface without hydrolysis even after 2 hrs gastric digestion. Similar results were observed for the proteins present in the serum phase. Both β-lg and α-La were hydrolysed within 1 min and β-lg remained unchanged along with incubation time after 1 min. However, small molecular weight peptides appeared increasingly as a broad diffuse band as the digestion time increased (Figure 6.5B). The other protein bands of high molecular weights of minor whey proteins including BSA were also seen to be degraded rapidly within the first 1 min by pepsin. As for the extent of hydrolysis of β-Lg, it should be noted that the hydrolysis of β-lg present in both the cream and serum phases did not occur continually over time during 2 hrs of the gastric digestion, suggesting its hydrolysis to a certain extent only at the beginning of digestion.

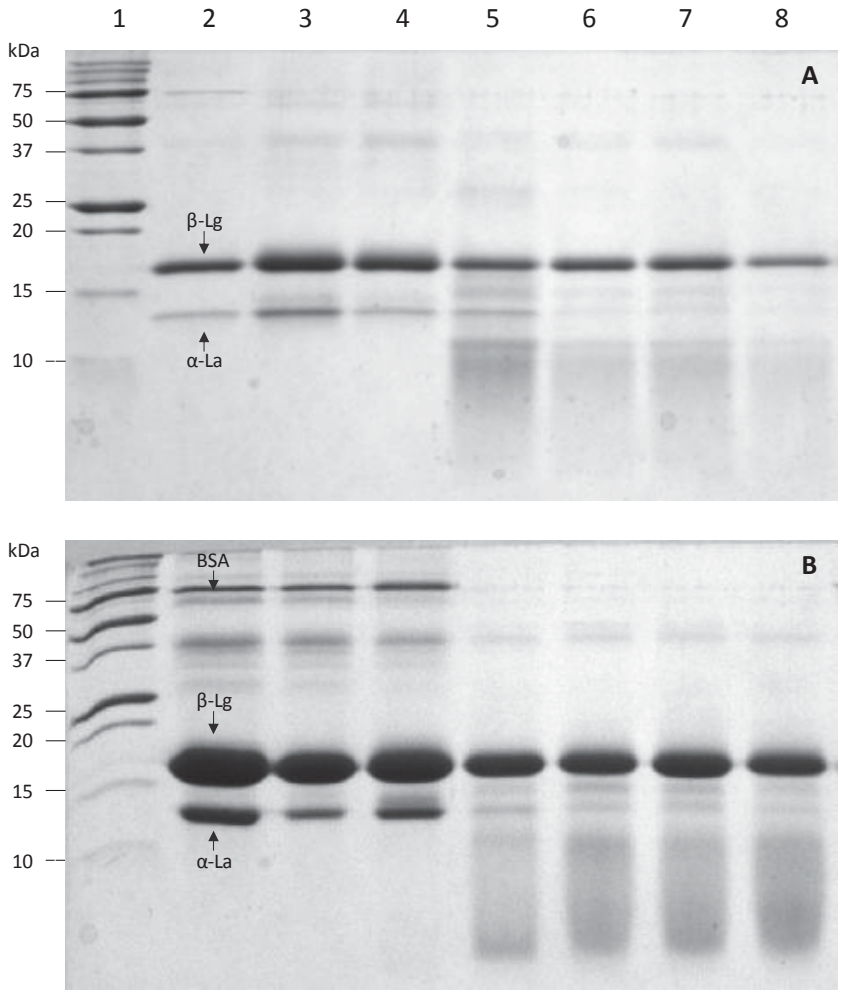


Figure 6.5 SDS-PAGE of WPI-stabilized emulsions during digestion in SGF for 2 hrs. Cream phase of emulsions (A); Serum phase of emulsions (B). Lanes from left to right: Molecular weight markers (Lane 1); 1% WPI protein (Lane 2); WPI emulsion mixed with RO water in 1:1 ratio (0.5% WPI protein) (Lane 3); WPI emulsion (1 % WPI protein) mixed with SGF in 1:1 ratio containing no enzyme (Lane 4), containing pepsin after digestion for 1 min (Lane 5), 10 min (Lane 6), 30 min (Lane 7) and 2 hrs (Lane 8).

It has been shown that α-La in solution is susceptible to pepsin hydrolysis in acidic conditions (Kim et al., 2007, Malaki Nik et al., 2010b), (Hur et al., 2009, Malaki Nik et al., 2011, Guo et al., 1995). β-lg in aqueous solution is however reported to be resistant to hydrolysis by pepsin under gastric conditions but it can be readily hydrolysed when it exists as adsorbed interfacial proteins. This has been explained to be due to changes in its conformational structure resulting from unfolding and rearrangement when it is adsorbed at the interface, thus exposing the enzyme binding site of β-lg from its protein core and facilitating the cleavage of β-lg by pepsin (Sarkar et al., 2009b, Molinari et al., 1996, Malaki Nik et al., 2010b). A recent study has pointed out that although β-lg or WPI-stabilized emulsions can remain stable in gastric environments, extensive

hydrolysis of adsorbed protein layers by pepsin results in destabilization and aggregation of the emulsion droplets (Malaki Nik et al., 2010b). This phenomenon was not observed in this study because of the relative mild hydrolysis of adsorbed β-lg at the interface. Malaki Nik et al. (2011) also showed that the stability of WPI emulsions, in terms of aggregation and coalescence induced by peptic hydrolysis during gastric digestion, could be significantly improved by increasing the concentration of WPI used in the preparation of emulsions from 0.5% to 1.5%, depending on the amount of unadsorbed protein present in the emulsion. They suggested that the pepsin hydrolysis of adsorbed proteins may render the adsorption of unadsorbed excess proteins to the interface, thus the emulsion stability can be maintained without droplet aggregation and coalescence (Malaki Nik et al., 2011).

6.3.2 WPI emulsion behaviour in SIF

6.3.2.1 Droplet size distribution and microstructure images

Unlike the WPI-stabilised emulsions were observed to be stable without pronounced changes in their size and properties during gastric digestion, the intestinal digestion with SIF (pH 7.5) containing bile extract and pancreatin resulted in significant changes in the droplet size and size distribution (Figs. 6.6 and 6.7). A noticeable change was observed, especially in the samples that had been digested for 30 min or longer times. After 30 min, the mean particle diameters, $d_{3,2}$ and $d_{4,3}$, increased sharply to 7.88 ± 4.87 μm and 48.57 ± 5.67 μm, respectively (Fig. 6.6). The particle size distribution changed from monodal to multimodal which had a broader and larger size range with a small peak at around 0.16 μm and a large peak at around 70 μm (Fig. 6.7), indicating significant coalescence of droplets. A relatively large increase in both $d_{4,3}$ and $d_{3,2}$ relative to the original emulsion was due to the formation of a large fraction of large particles (81 ± 5.2% droplets with diameter > 10 μm) in the particle size distribution. For the emulsions digested for 60 min and 120 min, the volume fraction of droplet diameter greater than 10 μm was 64 ± 0.2 % and 75 ± 15 %, respectively.

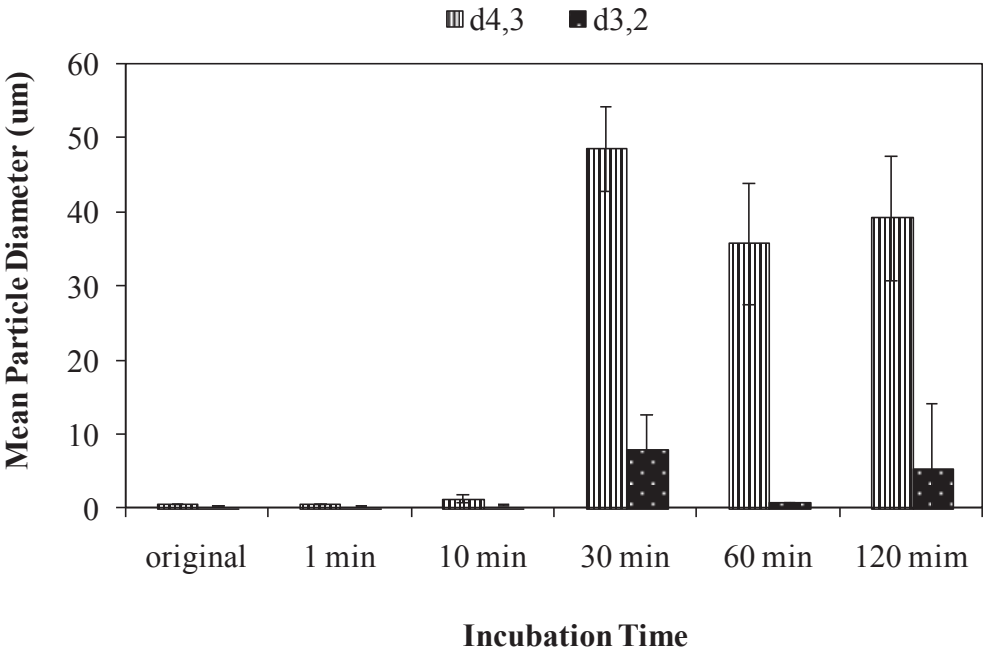


Figure 6.6 Mean particle diameters $d_{4,3}$ (μm) and $d_{3,2}$ (μm) of WPI-stabilized emulsions during digestion in SIF (containing pancreatin) over 2 hrs as a function of incubation time.

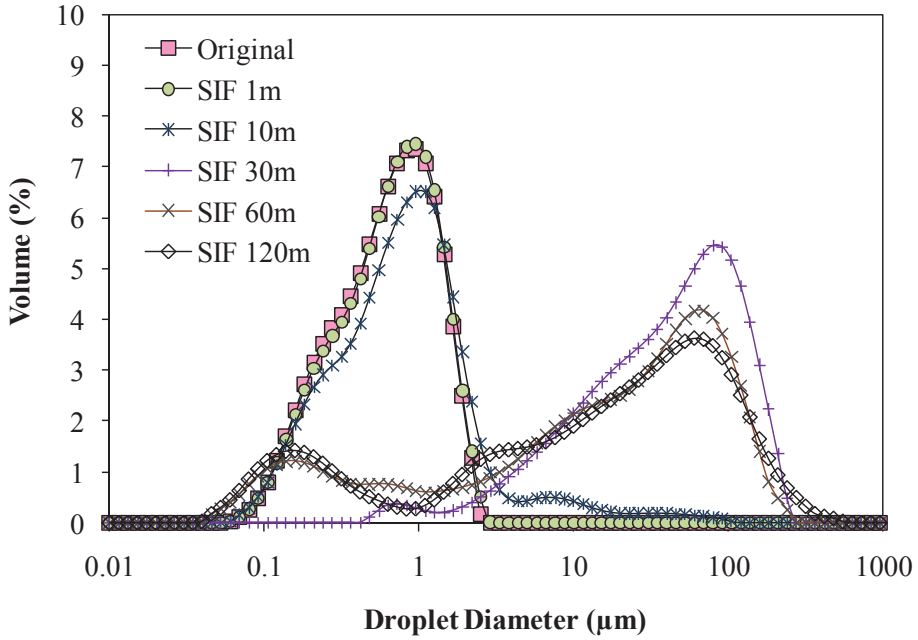


Figure 6.7 Size distributions of WPI emulsions (20 wt% soy oil and 1.0 wt% WPI) during digestion in SIF (pH 7.5, pancreatin and bile salts) as a function of time.

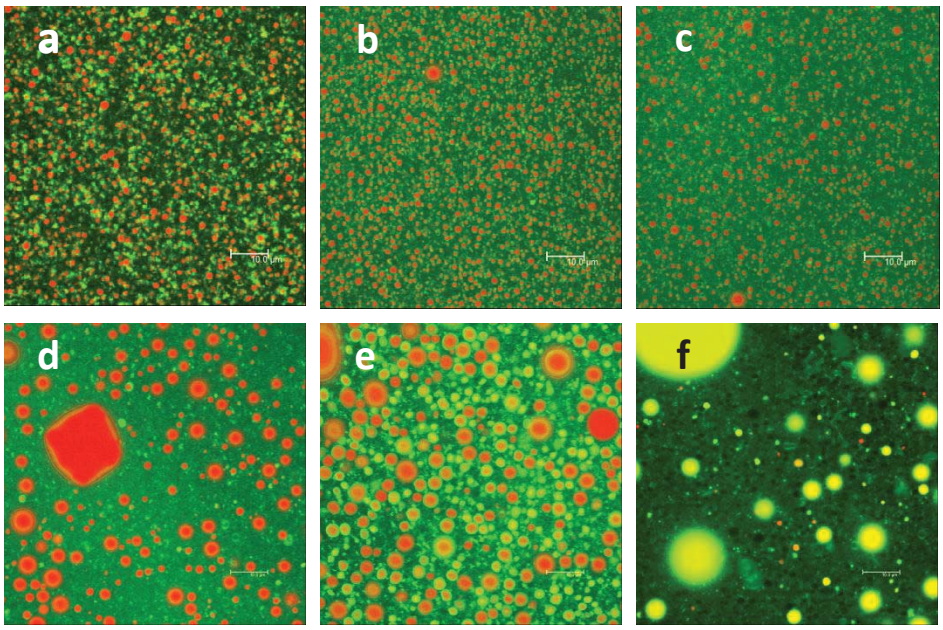


Figure 6.8 Confocal laser scanning microscope (CLSM) images of WPI-stabilized emulsions during digestion in SIF for 2 hrs. Original WPI-stabilized emulsion (a), WP-stabilized emulsions digested in SIF containing pancreatin and bile salts for 1 min (b), 10 min (c), 30 min (d), 60 min (e) and 2 hrs (f). The scale bar represents 10 μm.

The confocal microscopy images of WPI emulsions before and after SIF digestion, shown in Figure 6.8, illustrated the presence of large droplets formed after 30 min of SIF digestion, suggesting that there was extensive droplet coalescence. The change in the size of droplets due to their coalescence can be attributed to a combined effect of pancreatin and bile extract present in SIF. It has been suggested that the droplet coalescence is promoted by bile extract which displace adsorbed whey proteins (e.g. β-Lg) at the interface. This facilitates access and binding of pancreatic lipase to the emulsified core of lipid molecules, thus promoting the hydrolysis and break down of lipids into free fatty acids and mono-glycerides at the droplet surfaces which can displace adsorbed proteins from the droplet surfaces and thus destabilise the resulting emulsions (Mun et al., 2007, Gargouri et al., 1983). This is because free fatty acid and mono-glyceride substances are not effective at stabilizing oil-in-water emulsions against coalescence because of their lipophilic surface active characteristics.

Upon mixing with SIF containing bile extract and pancreatin and adjusting to pH 7.5, the ζ-potential of WPI emulsions at pH 7.2 decreased from -56.83 ± 2.3 mV to -31.6 ± 0.71 mV (Fig. 6.4). This value remained constant without a significant further change over time during 2 hrs of digestion. The decreased ζ-potential upon the addition of SIF

might have been due to an increased ionic strength of the emulsion/SIF mixture by salts present in SIF which contained 50 mM NaCl and 30 mM $CaCl_2$. In addition, bile extract might also have contained some salts. Moreover, the adsorption and displacement of pancreatin on the interfacial proteins could possibly alter the ζ-potential (Mun et al., 2007).

6.3.3 WPI emulsions behaviour in simulated gastrointestinal tract

6.3.3.1 Average droplet size

The effect of sequential digestion on the size and properties of WPI-stabilized emulsions was also studied. Figures 6.9 and 6.10 show the mean particle diameters and particle size distributions of WPI emulsions digested in SIF for 1, 10, 30, 60 and 120 min after they had been incubated with SGF for 5 min (in the absence of pepsin) and 1 min, 30 min and 2 hrs (in the presence of pepsin).

When WPI emulsions were incubated in SIF after SGF digestion, the changes occurring in the size and particle size distribution were observed to be more or less similar to those of the emulsions subjected directly to SIF without SGF. When WPI emulsions were mixed with SGF containing no pepsin and further treated with SIF, a drastic increase in the droplet size ($d_{4,3}$) was observed from 0.96 to 60.01 μm after 2 hrs digestion with enzyme pancreatin and bile extract (Fig. 6.9). This result could be due to the aforementioned explanation on the combined effect of pancreatin and bile extract resulting in the generation of free fatty acids (Hur et al., 2009, Malaki Nik et al., 2010a, Malaki Nik et al., 2010b, Mun et al., 2007). Similar trends were also obtained for the emulsions treated first with SGF containing pepsin for 1, 30 and 120 min and then further digested with SIF (Fig. 6.9). The emulsion droplet size $d_{4,3}$ increased to a very large size range of 55- 72 μm, depending on the gastric digestion time applied prior to the intestinal digestion, which was bigger than the emulsions that had been digested in SIF only. These results were able to be confirmed by the results of the confocal microscopic examination and particle size distribution measurement (Figs. 6.10 and 6.12).

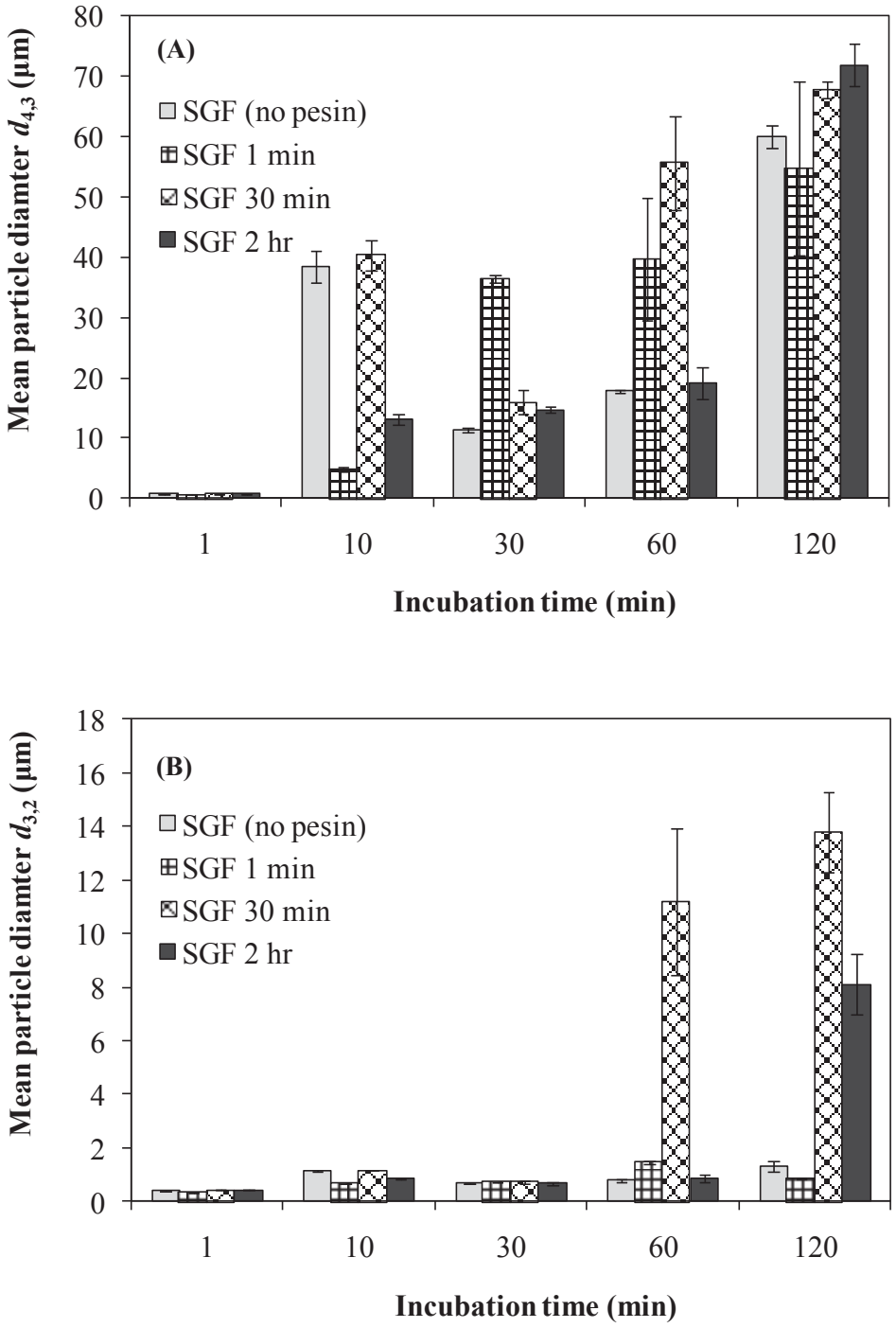


Figure 6.9 Mean particle diameters $d_{4,3}$ (μm) (A) and $d_{3,2}$ (μm) (B) of WPI-stabilized emulsions during digestion in SIF (containing pancreatin) over 2 hrs after gastric treatment as a function of incubation time.

At the end of 2 hrs of digestion, the mean particle diameter ($d_{4,3}$) of all the emulsions treated with both SGF and SIF appeared to be larger than that of the emulsions digested with SIF only without SGF, regardless of the presence of pepsin in SGF. Also, the gastric digestion times applied prior to the SIF digestion seemed to have no significant

influence on the subsequent emulsion digestion in SIF. These observations suggest that the peptic enzyme in SGF had no significant effect on the subsequent intestinal digestion but the sequential SGF/SIF digestion might have weakened further the stability of WPI emulsions. The possible reasons could be due to the exposure of WPI emulsions to more dramatic environmental changes, including pH changes from its original pH 7.2 to pH 1.6 and then to pH 7.5 and ionic strength changes due to different salt concentrations between SGF and SIF. These changes might have partly contributed to alteration to the properties and conformational structures of adsorbed whey proteins at the interface.

Regarding the data shown in Figures 6.9 and 6.10, it should be mentioned that the patterns of changes in the particle diameters and particle size distributions between the emulsion samples in response to their respective digestion times were not consistent and had some fluctuations. The discrepancy in some of the results obtained in this study could be due to some sampling errors because the emulsions were not homogenous with some free oil and large droplets resulting from the extensive emulsion droplet coalescence and destabilization. Consequently, some samples collected at different time intervals for their analysis might have not been so representative, hence causing some variations in the particle size measurement results. Figure 6.11 shows the images of emulsion samples collected during digestion which were then centrifuged to qualitatively monitor and compare the extent of destabilization that had occurred between samples. For the emulsions digested with SGF only (Fig. 6.11A), the presence of free oil was not detected whereas all the emulsions subjected to the SIF digestion for longer than 30 min showed some free oil present besides a layer of cream. Furthermore, the appearance of colour and optical properties of the subnatant emulsion phase in these samples appeared more yellow-greenish and less opaque with increasing digestion time, suggesting there were more extensive emulsion breakdown and destabilization.

6.3.3.2 Size distribution and microstructure

The intestinal digestion of WPI emulsions after the SGF treatment altered the size distributions and microstructures of emulsions significantly, regardless of the SGF digestion time and the presence of pepsin in SGF prior to SIF digestion (Figs. 6.10 and 6.12). The average droplet diameter increased drastically with a multimodal size

distribution. However, in the absence of pancreatin in SIF, the size distribution of emulsions remained monomodal with a peak at around 1.0 μm (data not shown). These results suggested that coalescence occurred in the emulsions and that it was promoted by pancreatin. Bile extract could have displaced the rest of β-lg proteins from the interface, allowing the ease access for pancreatin to hydrolyze triglycerides, leading to the generation of free fatty acids (Hur et al., 2009, Mun et al., 2007, Mun et al., 2006, Sarkar et al., 2009c). This assumption was verified by confocal microscopic image results. As mentioned earlier, a layer of free oil was observed on the top layer of emulsions after 30 min intestinal digestion after gastric treatment.

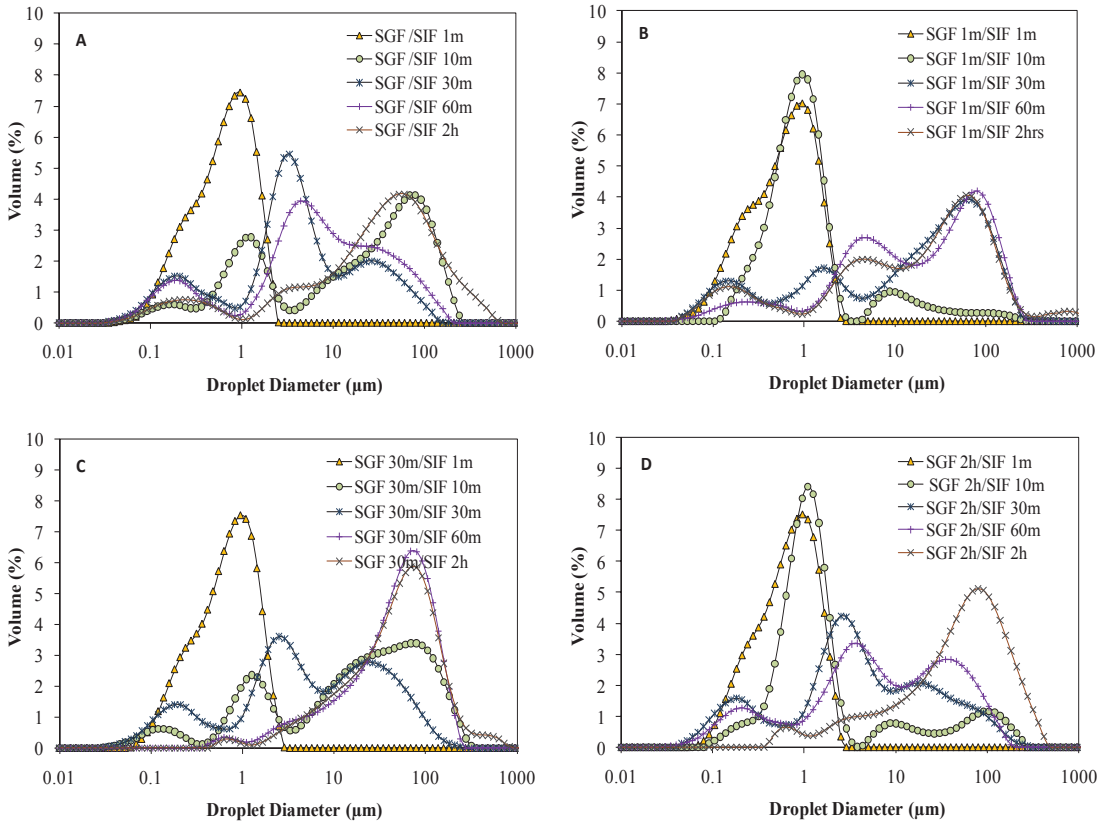


Figure 6.10 Particle size distributions of WPI-stabilized emulsions after sequential digestion with both SGF and SIF. (A) after mixing with SGF (without pepsin stirring for 5 minutes); (B), (C) and (D) after mixing with SGF (containing pepsin) for 1, 30 and 120 min, respectively.

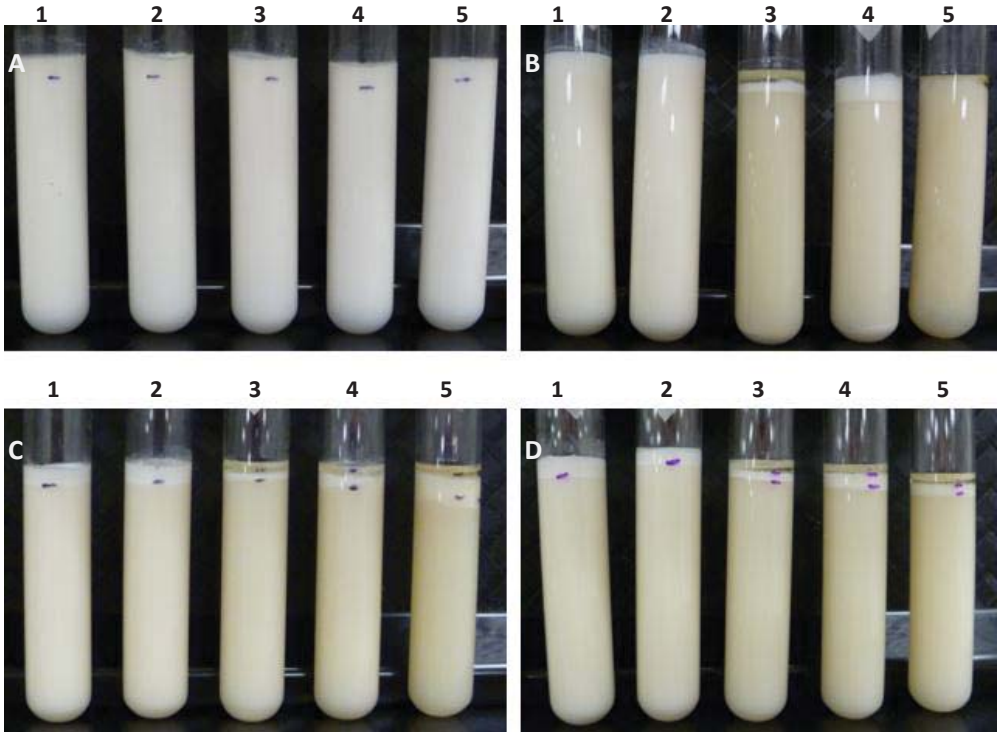


Figure 6.11 Images of emulsions taken over time during digestion. (A) SGF for 2 hrs , (B) SIF for 2 hrs, (C) SGF 30 min/SIF 1 min -2 hrs and (D) SGF 2 hrs/SIF 1 min – 2 hrs. Numbers from 1 to 5 represent 1 min, 10 min, 30 min, 1 hr and 2 hrs incubation times, respectively.

The microstructures of emulsions examined using confocal laser scanning microscopy (CLSM) confirmed the size distribution results (Fig. 6.10). It is apparent that WPI emulsion droplets increased along with 2 hrs intestinal digestion time. As previously shown in Figure 6.7, when WPI emulsions were treated only with SIF, the big droplets began to appear, especially after 30 min. The treatment with SGF (Fig. 6.12) accelerated the occurrence of coalescence during the subsequent intestinal digestion as bigger droplets were observed within 10 min intestinal digestion (with pancreatin) after the gastric treatment. This was probably due to the interface of WPI emulsions had been already weakened or disrupted after gastric treatment, allowing ease access of enzymes or surfactants when they were further treated with SIF. These results were in agreement with the previous study (Malaki Nik et al., 2010b).

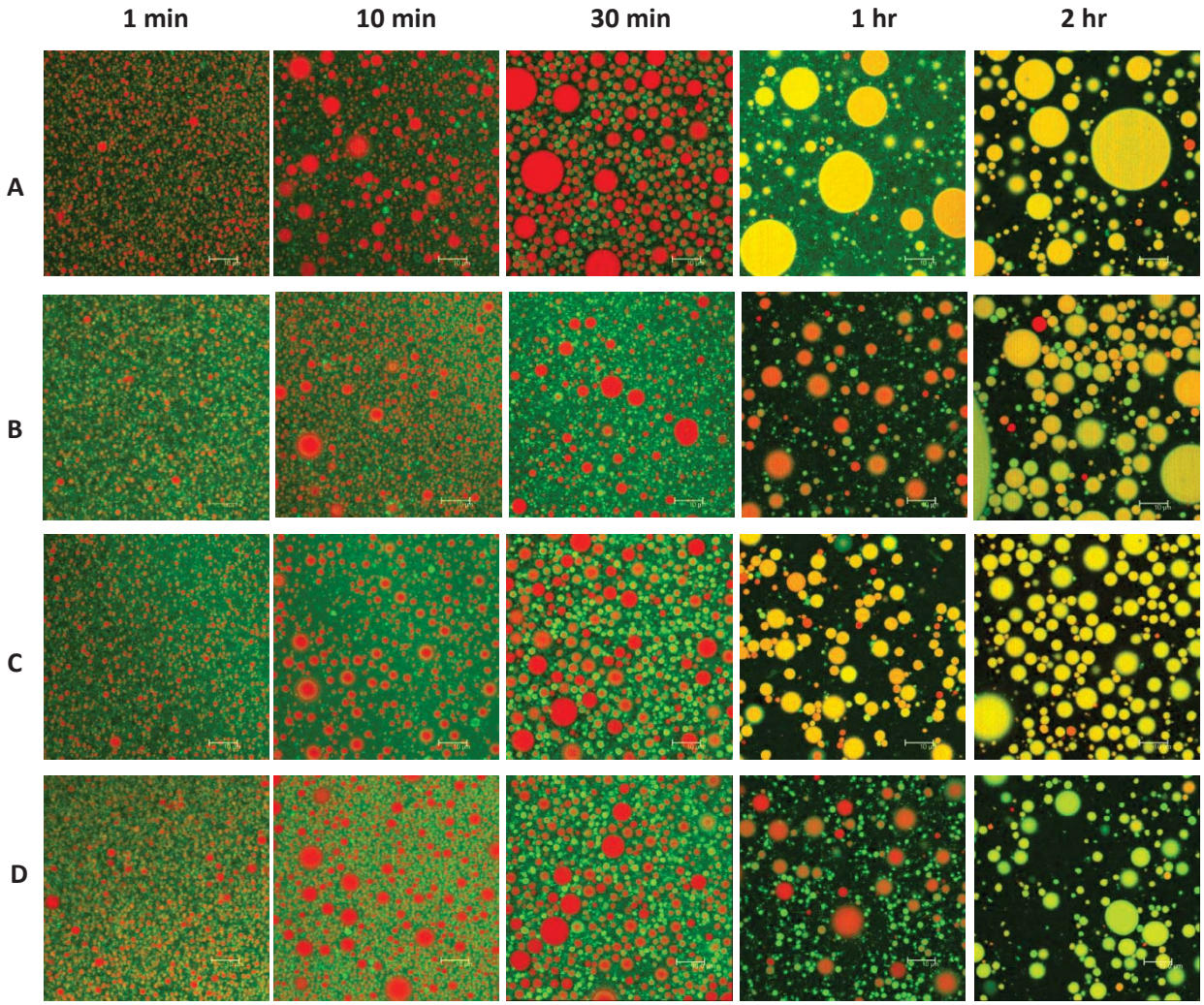


Figure 6.12 Confocal laser scanning microscope (CLSM) images of WPI-stabilized emulsions during the subsequent digestion in SIF for 1 min - 2 hrs (A) after digestion with SGF (without pepsin stirring for 5 minutes); (B), (C) and (D) after digestion with SGF (containing pepsin) for 1, 30 and 120 min, respectively. The scale bar represents 10 μm.

6.3.3.3 Zeta potential

The ζ-potentials of WPI emulsions were measured after treatment first with SGF (1, 30 and 120 min) and then SIF (1, 10, 30, 60 and 120 min). The results are shown in Figure 6.13. As already described in the above, the ζ-potential decreased from -56.8 ± 2.3 mV to -20.3 ± 1.4 mV when the original WPI emulsions were treated directly with SIF containing no pancreatin. In the presence of pancreatin, the extent of initial decrease was observed to be significantly less being as -31.6 ± 0.7 mV. When the emulsions were mixed with SIF after the SGF treatment, the negative ζ-potential values were observed to be slightly higher than those of the corresponding emulsions treated for the same time

only with SIF (1, 10, 30, 60 and 120 min). After 1 min, the ζ-potential values were all around -36.2 ± 1.6 mV, regardless of the SGF digestion time applied prior to the intestinal digestion. This value seemed to increase significantly after 10 min up to -45.4 ± 2.1 mV and then remained relatively constant afterwards being around -49.4 ± 2.63 mV. The increase in the negative charge of ζ-potential could be possibly due to the interfacial layer composition change occurring in the presence of bile extract and pancreatin (Mun et al., 2007). As described above, the SDS PAGE results (Fig.6.5) showed that α-LA was rapidly digested in SGF after 1 min and only a portion of β-lg remained at the interface together with hydrolyzed peptides. Therefore, the increase in the negative charge could be due to the hydrolysis of interfacial layer proteins coupled with the displacement of β-lg by anionic bile extract and enzyme pancreatin. This assumption could be supported by the study reported by Hur et al. (2009) that bile extract has the capabilities of adsorbing rapidly at the interface and also penetrating into and fully displacing β-lg from an oil-water interface. Moreover, bile extract was also reported to facilitate the accessibility of pancreatin to the emulsified lipid core, resulting in the generation of free fatty acids (Mun et al., 2007). Finally, similar to the results of NaCas-stabilized emulsions described in Chapter 4, the zeta potentials were not significantly different during the subsequent intestinal digestion between the WPI emulsions that had been digested in SGF with or without pepsin (data not shown).

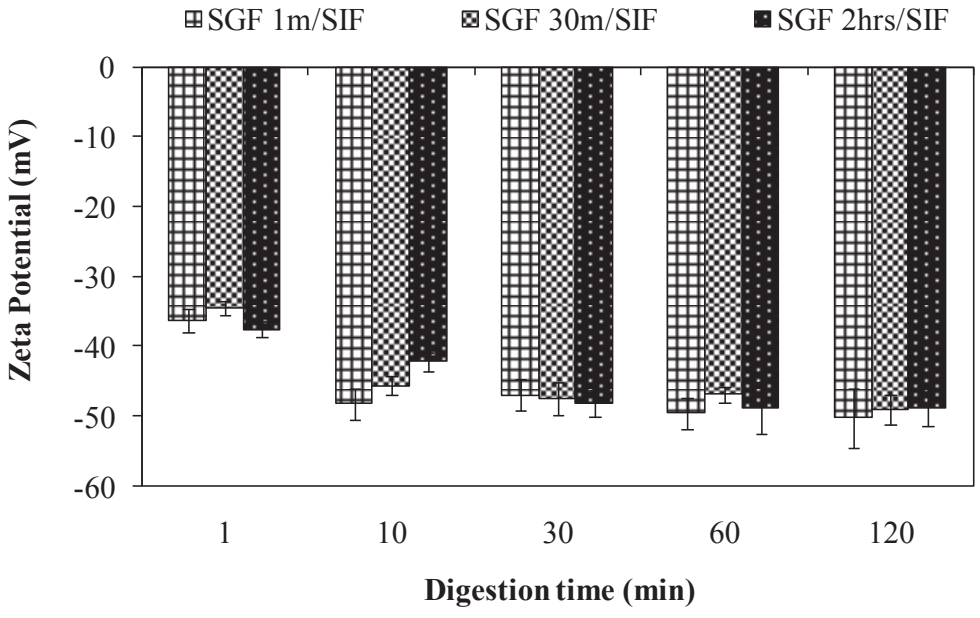


Figure 6.13 The ζ-potentials of WPI-stabilized emulsions measured during digestion with SIF for 2 hrs after gastric treatment for different incubation times (1 min, 30 min and 2 hrs).

6.3.3.4 Free fatty acid release

The amount of free fatty acids hydrolysed from emulsified lipids was determined by pH-stat titration during 2 hrs of digestion in SIF containing pancreatin with or without bile extract after they had been digested in SGF for 1, 30 or 120 min, respectively. The WPI emulsions were also titrated during incubation in SIF without gastric digestion (Fig. 6.14). The results show that the emulsified lipids were hydrolyzed rapidly within the first 20 min of digestion, regardless of the gastric digestion time and that the hydrolysis rate of fatty acids was much faster in the presence of bile extract. A fairly high rate of free fatty acid release was observed to occur up to 60 min and thereafter the rate of hydrolysis declined significantly in both cases of the presence and absence of bile extract.

A similar result was obtained for the emulsions that were digested in SIF without gastric digestion. A noticeable difference was that the hydrolysis rate of fatty acids appeared to be slightly lower during the first 1 hr digestion but the difference was not observed at the end of 2 hrs digestion. This indicates that the intestinal digestion of emulsified lipids may not have been affected greatly by the gastric digestion prior to the intestinal digestion. The slight faster hydrolysis rate of of fatty acids from the samples digested in both SGF and SIF at the initial incubation of 1 hr might have been due to the effect of hydrolysis of α-LA and a partial hydrolysis of β-lg from the interfacial layer by pepsin in SGF as shown in the SDS-PAGE results (Fig. 6.5) so it might have been easier for pancreatin lipase to access and hydrolyse the lipid core (Malaki Nik et al., 2010b).

The amount of free fatty acids hydrolysed after 2 hrs intestinal digestion in the presence of bile extract was around 70 μmol per mL emulsion in all samples that had been treated with SGF for different times. On the other hand, in the absence of bile extract, the amount of free fatty acids hydrolysed was much lower at about 48 μmol per mL emulsion. This indicated that bile extract had a significant influence on the hydrolysis of emulsified lipids, promoting their hydrolysis significantly. As described earlier, it is known that bile extract displaces β-lg from the interface or directly adsorbs onto the interface. This facilitates the binding and access of pancreatin to the lipid core for

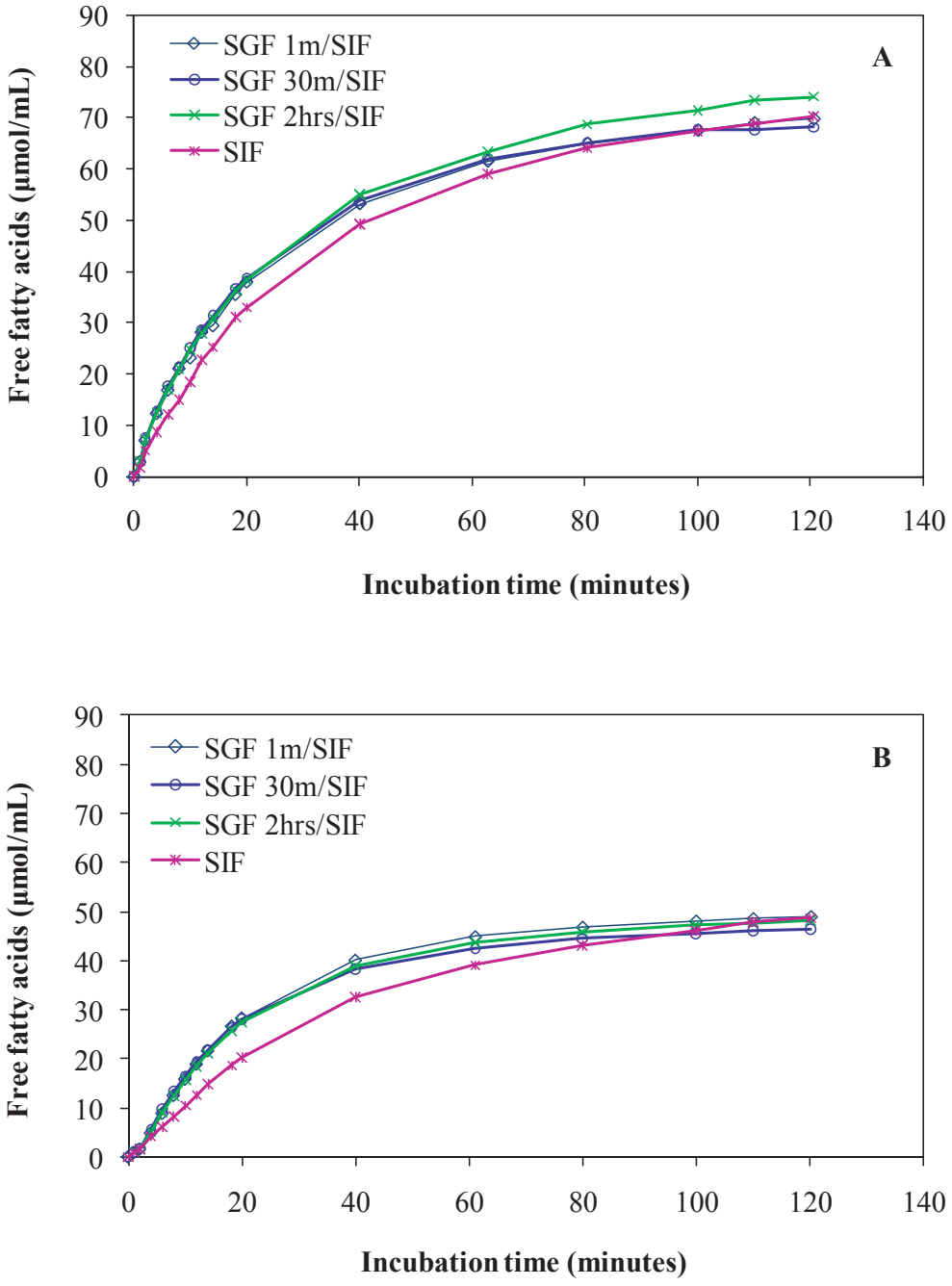


Figure 6.14 The release of free fatty acids (µmol/mL emulsion) hydrolyzed from WPI-emulsions during digestion in SIF for 2 hrs after gastric treatment for different times. (A) SIF containing 5.0 mg/mL bile salts and 1.6 mg/mL pancreatin and (B) SIF containing 1.6 mg/mL pancreatin and no bile salts.

fatty acids released was similar between WPI-stabilized emulsions and sodium caseinate-stabilized emulsions. They also reported that the hydrolysis rate of fatty acids was rapid within the first 20 min and then slowed down afterwards (Malaki Nik et al., 2011). A similar trend was observed in our study that there was a rapid increase of free

fatty acids released within the first 30 min followed by a relatively small increase afterwards.

6.4 Conclusions

This study provides an insight into the lipid hydrolysis of WPI stabilized O/W emulsions as well as its physicochemical changes during sequential digestion in SIF after pre-gastric treatment for different times.

During gastric digestion, α-LA adsorbed at the interface was fully hydrolyzed by pepsin after 2 hrs while a portion of β-lg proteins of WPI emulsions showed resistance to enzymatic hydrolysis under gastric conditions, and no pronounced changes in droplet size and microstructure was observed. When WPI emulsions were treated with SIF, there was a great increase in droplet size and microstructure after 30 minutes digestion. During the sequential digestion of WPI stabilized emulsions in SIF after pre-gastric treatment, more extensive coalescence were observed after a shorter time -10 minutes of intestinal digestion resulting in a more significant change in droplet size and microstructures. These results indicate that the treatment with SGF accelerated the occurrence of coalescence of WPI emulsions during their subsequent digestion in SIF. Similar to that of sodium caseinate and Tween 20-stabilized emulsions, the pre-gastric treatment prior to the intestinal digestion had no pronounced influence on the quantity of free fatty acid released and the presence of bile extract was also found to significantly increase the rate and extent of lipid hydrolysis.

Chapter 7. Conclusions

This study provides a valuable insight into understanding the emulsion digestion and lipid hydrolysis occurring during digestion in SGF or SIF and during sequential digestion in SGF and SIF. During gastric digestion, the emulsions stabilized by sodium caseinate were shown to undergo significant droplet aggregation and coalescence, resulting in a significant increase in the size of oil droplets. On the other hand, the emulsions stabilized with WPI or Tween 20 showed no pronounced changes over time during 2 hrs of gastric digestion. When sodium caseinate and WPI-stabilized emulsions were further exposed to SIF after gastric digestion, these emulsions had great further changes in their physicochemical properties, whereas Tween 20 only showed some extent of coalescence mainly after 2 hrs of the subsequent intestinal digestion. The occurrence of coalescence of oil droplets in SIF was attributed to the effect of bile extract that displaces proteins or surfactants from the interface. This facilitates the adsorption of pancreatin to the interface, thus promoting the hydrolysis of lipids and leading to the disruption and coalescence of oil droplets. The amounts of free fatty acids released over time during digestion in SIF from three different types of emulsions were similar regardless of the gastric digestion applied prior to the intestinal digestion. This indicates that although the digestion sequentially in SGF and SIF had a significant further influence on changes in the size of oil droplets, it did not affect the rate of lipolysis, and the lipid hydrolysis was not affected by types of emulsifiers.

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Appendices

Appendix 1: Solutions and chemicals used in SDS-PAGE

1.5 M Tris-HCl buffer (resolving gel buffer), pH 8.8

18.15 g Tris was dissolved in about 60 mL milli-Q water. The pH was adjusted to 8.8 with 6 M HCl and made up to 100 mL volume. The buffer was stored at 4^{o}C.

0.5 M Tris-HCl buffer (resolving gel buffer), pH 6.8

6.05 g Tris was dissolved in about 60 mL milli-Q water. The pH was adjusted to 6.8 with 6 M HCl and made up to 100 mL volume. The buffer was stored at 4^{o}C.

10% Sodium Dodecyl Sulfate (SDS)

10 g of SDS was dissolved in mlli-Q water with gentle stirring, and made up to volume 100 mL. The solution was stored at room temperature.

10% Ammonium Persulphate (APS)

200 mg APS was dissolved in 2 mL milli-Q water.

0.1% Bromophenol blue solution

0.08 g bromophenol blue solution was mixed with 0.35mL 0.1 M NaOH, and diluted to 80 mL with water.

Staining Solution-Coomassie Brilliant Blue Solution (0.3%)

3.0 g of Coomassie Brilliant Blue R was dissolved in a mixture of 700 mL water, 200 mL Isopropanol and 100 mL glacial acetic acid. The solution was then filtered through Whatman filter paper (Grade 4) and stored in a dark bottle.

Destaining solution

100 mL of isopropanol and 100 mL of glacial acetic acid were added to 800 mL of water and stored in a well closed container.

Sample buffer

Sample buffer was prepared by mixing 3.125 mL 0.5M Tris-HCl buffer (pH 6.8), 5 mL 10% SDS, 6.25 mL glycerol, 2.5 mL 0.1% bromophenol blue and made up to 25mL with Milli-Q water. 2% β-mercaptoethanol was added into the sample buffer prior to use. The use of SDS was to provide the same net negative charge to protein molecules so that the proteins were separated based on their molecular weights rather than their initial charges. An addition of 2% β-mercaptoethanol was to reduce the disulphide linkages between protein molecules.

5X Electrode buffer

Electrode buffer (5X) was prepared by dissolving 7.5g Tris, 36g glycine, and 2.5g SDS powder in milli-Q water, finally made up to 1 L. The pH of the stock solution was about 8.6. The solution was then diluted with milli-Q water in the ratio of 1:4 before use.

Resolving gel (16%)

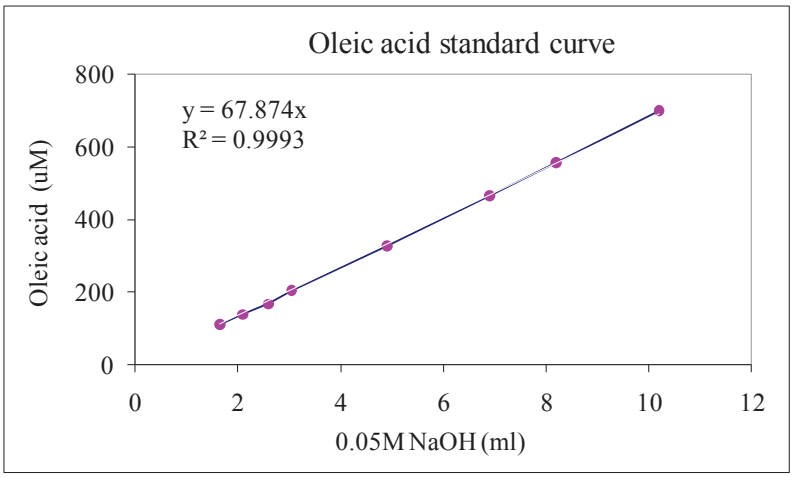
A 10 mL of resolving gel buffer was made up of 2.02 mL milli-Q water, 2.5 mL 1.5 M Tris-HCl buffer, 100 µL 10% SDS solution and 5.3 mL 30% acrylamide/bis mixture. The mixture was then degassed under vacuum for 15 minutes. After degassing, 5 µL TEMED (tetramethylethylenediamine) and 50 µL freshly prepared 10% (w/v) APS (ammonium persulphate) were added. The final mixture was then loaded between the pair of electrophoresis glass plates. A small quantity of deionized water was added to form an upper layer to prevent drying out of the gel. The gel was kept for 1 hour for polymerisation at room temperature. The water was removed with a syringe and finally with filter paper before adding the stacking gel solutions.

Stacking gel preparation (4%)

A 10 mL stacking gel buffer contained 3.05 mL milli-Q water, 1.25 mL 0.5 M Tris-HCl buffer, 50 µL 10% SDS solution and 800 µL 30% acrylamide/bis mixture. The mixture was degassed under vacuum for 15 minutes. 5 µL TEMED (tetramethylethylenediamine) and 25 µL freshly prepared 10% (w/v) APS were added. The final mixture was then poured onto the top of the resolving gel. A 0.75 mm plastic comb with 10 slots was placed at the top of stacking gel solution to form loading slots. The gels were left for

complete polymerization for 1 hour at room temperature. The prepared gels were then placed into a sealed container and stored at 4°C until use.

Appendix 2: Oleic acid standard curve for free fatty acid release calculation



Appendix 3:

The calculation of free fatty acids released if emulsified lipid triglycerides were fully hydrolysed:

$M_{oleic\ acid} = 881$ mol/L

Since hydrolysis of 1 mol triglyceride will release 2 mols of free fatty acids, thus the amount of free fatty acids released is:

$$\frac{m_{triglyceride} \times oil\%}{M_{triglyceride}} \times 2 \times 1000 = \frac{20\% \times 1}{881} \times 2 \times 1000 = 454\ \mu mol/mL$$