IN VITRO DIGESTION AND BETA-CAROTENE DELIVERY OF EMULSION STABILIZED BY HEATED WPI AND PECTIN MIXTURE

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By

Yafan Tang

Dr. Bongkosh Vardhanabhuti, Thesis Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

IN VITRO DIGESTION AND BETA-CAROTENE DELIVERY OF EMULSION STABILIZED BY HEATED WPI AND PECTIN MIXTURE

Presented by Yafan Tang

a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Bongkosh Vardhanabhuti, Department of Food Science

Dr. Mengshi Lin, Department of Food Science

Dr. Xiaoqin Zou, Department of Biochemistry

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***IN VITRO* DIGESTION AND BETA-CAROTENE DELIVERY OF EMULSION STABILIZED BY HEATED WPI AND PECTIN MIXTURE**

Yafan Tang

Dr. Bongkosh Vardhanabhuti, Thesis supervisor

ABSTRACT

In this study, the impact of a whey protein isolate (WPI) and pectin complex (formed heating the mixture at 85℃ and pH 7) on digestion properties and delivery of β-carotene in oil-in-water emulsions was investigated using the *in vitro* gastric and intestinal models. Beta-Carotene enriched emulsions were stabilized by WPI, heated WPI (HWPI), unheated WPI-pectin mixture (MIX) or heated WPI-pectin mixture (HMIX). As emulsions were digested in the *in-vitro* gastric and intestinal digestions, their droplet size, zeta-potential, microstructure, free fatty acid release and beta-carotene release were measured. Results showed that emulsions stabilized by WPI underwent significant changes during gastric digestion as shown by increased mean droplet sizes and extensive coalescence. These changes have been found to decrease bioaccessibility of beta carotene. The presence of pectin could lead to oil droplet flocculation with much lower extent of coalescence which resulted in decreased beta-carotene release and thus favoring its bioaccessibility during intestinal digestion. Heating of WPI or mixed WPI and pectin also led to decreased coalescence and provided protective effect on beta carotene during gastric digestion. During intestinal digestion, emulsions stabilized by WPI and HWPI completely broke down due to proteolysis while those containing pectin showed better stability due to a more intact interfacial layer structure. Generally, it was found that pectin and heating process favored the stability of emulsion during gastrointestinal digestion overall, which

facilitated the digestion of emulsified lipid. However, at the same time, the presence of pectin inhibited the intestinal digestion of lipid by limiting the adsorption of lipase at the interface. Similar effects were observed on the release and bioaccessibility of beta-carotene. A linear relationship was found between beta-carotene bioaccessibility and lipid digestibility. In conclusion, heated WPI and pectin soluble aggregates can be utilized and optimized in designing the delivery and bioaccessibility of lipid and beta carotene.

CHAPTER 1 INTRODUCTION

1.1 Whey protein and its functionality in food emulsion

Oil-in-water emulsions are utilized in numerous commercial foods and food ingredients, including desserts, dips, ice cream, coffee, sauces, dressings, beverages and soups. In emulsion-based food products, the most important functional ingredient is the emulsifier, as the properties of the oil-water interface often determine the stability and processing behavior of the emulsions. The most common emulsifiers used in the food industry are amphiphilic proteins, phospholipids, and small molecular weight surfactants. Concerned with clean label and nutritional aspect, customers are avoiding artificial surfactants, resulting in the search for natural emulsifiers with improved emulsification and stabilization properties. Naturally, whey protein becomes one of the most popular emulsifiers in the food industry in recent years, due to its excellent functional properties and high nutritional value.

Once a by-product of cheese manufacturing, whey protein is a mixture of globular proteins with two main fractions: 65% of β-lactoglobulin, and 25% of alpha-lactalbumin. The three common commercial whey protein products available in the market are whey protein isolate (WPI), whey protein concentrate (WPC), and whey protein hydrolysate (WPH). The main difference between WPC and WPI is protein content, ranging from 35% to 89% and over 90%, respectively. WPH is more different from the other two, as it is a predigested and partially hydrolyzed whey protein, containing rich bioactive peptides.

With its excellent surface activity, whey proteins can quickly adsorb at the oil droplet interface where they unfold and form a stabilizing layer against oil droplet coalescence, droplet flocculation or creaming (Dickinson and James 1999; Dickinson and others 2001; Zhai and

others 2011). However, the interfacial layer formation and the accompanying structural rearrangements strongly depend on conditions of solution, such as: pH value, ionic strength, temperature and other ingredients in solution. Those are barriers limiting whey protein from extensive application in reality. Thus, nowadays many studies are being conducted to enhance the emulsifying capacity of WPI in extreme conditions to satisfy the need of food industry. These attempts can be summarized into three approaches: (1) covalently or non-covalently reacting with other molecules such as polysaccharides to produce larger biopolymer (Chevalier and others 2001; Losso and Nakai 2002); (2) causing non-covalent and/or covalent interactions among proteins to produce soluble aggregates or (3) hydrolyzing proteins to produce smaller hydrolysates and/or peptides (van der Ven and others 2001).

Previous work in our laboratory has focused on enhancing whey protein functionality through forming soluble complexes with polysaccharides. This study also focused on heat-induced WPI-pectin complex. Therefore, interaction between protein and polysaccharide is illustrated in next section.

1.2 Interaction between protein and polysaccharide

Polysaccharides are polymeric carbohydrate molecules, composed of long chains of monosaccharide units bound together by glycosidic linkages (Baghurst and others 1996). There are various kinds of polysaccharides in nature with variety of structures, from long structure chain to branched structure (Stephen and Phillips 2016; Elleuch and others 2011). Due to their unique chemical structure and numerous desirable physical properties, polysaccharides are widely used as thickener, stabilizer, texturizer, and health-promoting ingredients (Saha and Bhattacharya 2010; Dickinson 2009). Furthermore, an increasing interest is to utilize

polysaccharide to enhance protein surface functionality. In summary, polysaccharides can react with protein in two approaches. Firstly, protein and polysaccharide are linked together by a single covalent bond through Millard reaction and form a larger biopolymer called 'conjugate' (Kato 2002). Protein-polysaccharide conjugates have been found to have very effective emulsification and emulsion-stabilizing properties. However, this approach usually requires longtime high temperature heat treatment, which makes it less practical in mass manufacturing.

The other type of reaction is driven by electrostatic force (De Kruif and others 2004). Both proteins and polysaccharides carry net charge in aqueous solution. At pH value lower than protein PI, positively charged proteins and negatively charged polysaccharides can intensively bind together by strong attractive electrostatic force. Nevertheless, recent study found that heating whey protein and polysaccharide together at pH above the pI of the protein also can induce complex formation (Zhang and others 2014). Although both proteins and polysaccharides carry net negative charges, the positively charged patches on protein residues interact with anionic polysaccharides, altering protein aggregation and further modifying protein functionality (Girard and others 2003). Because mildness of this reaction, reactants concentration could be further enhanced without leading to aggregation, meeting the demands of industry and customer for high protein content food. In functionality aspect, previous work in our lab has shown that these soluble complexes can significantly enhance the stability of emulsion at pH near PI. However, the digestion property of this system has never been investigated, and its application in delivering lipophilic nutrient is worthy for exploring as well.

1.3 Digestion of emulsion and its application

So far, many efforts have been made towards the characterization of the physicochemical properties of emulsions during processing and storage. Recently, the changes in properties of emulsions during gastrointestinal (GI) digestion also attract much attention. Researchers are seeking how to control and optimize the extent of lipid hydrolysis during digestion and designing delivery systems with emulsion to protect and deliver bioactive lipophilic compounds.

As an emulsion passes through human GI tract, its composition and structure of the interfacial layer can be dramatically changed due to enzymes, change of pH, ionic strength and the presence of biosurfactants. Digestion of emulsified lipid and the release of carried bioactive lipophilic compounds are affected not only by the intrinsic factors such as oil composition, oil droplet size and native properties of bioactive compounds, but also the extrinsic factors such as composition of emulsifier and structure of initial interfacial layer structure (McClements and others 2007).

Many papers show that polysaccharides may significantly alter digestion of encapsulated lipid (Beysseriat and others 2006; Zhang and others 2015; Xu and others 2014b). Polysaccharides can bind with bile salt in intestine, retarding transportation of oil, and act as steric barrier on the oil droplet surface directly limiting absorption of lipase to the oil droplets. Furthermore, polysaccharide may alter digestion of lipid by affecting physical property of the emulsion in the GI tract. It has been shown that pectin can promote flocculation of oil droplet during gastric digest leading to appreciable effect on oil droplet size because of more possibility of coalescence. The presence of polysaccharides can also impact the digestion of protein-polysaccharide mixed systems. Considering the interactions between proteins and polysaccharides under certain conditions, the digestion profile of proteins and even the structure

of interfacial layer during digestion could be significantly altered, which may further influence digestibility of lipid and bioavailability of carried lipophilic bioactive compounds(Zhang and Vardhanabhuti 2014b).

In this study, WPI-pectin complex stabilized emulsions were digested in simulated human gastrointestinal tract. The changes of physiochemical properties of emulsions during digestion, digestibility of lipid and bioavailability of carried β-carotene were fully investigated by various characterization methods. The overall objective was to determine key factors affecting the digestion properties and β-carotene delivery of emulsions stabilized by mixed WPI and pectin.

To achieve this goal, several factors were investigated: i) heat treatment, ii) biopolymer concentration and ratio during complex formation, and iii) biopolymer concentrations in final emulsions.

Our ultimate goal is to develop a food-based delivery system to encapsulate, protect, and release bioactive components believed to benefit human health. By determining an effective approach to control lipid digestion in human body, food manufacturer can develop healthy food products that deliver maximum health benefits without reducing sensorial quality of eating.

CHPATER 2 LITERATURE REVIEW

2.1 Protein-polysaccharide complex

2.1.1 Formation of protein-polysaccharide complex

Proteins and polysaccharides are both natural biopolymers that are widely used in foods and beverages. In particular conditions, they can interact together and form complex. Complex formation is mainly driven by electrostatic force. Nevertheless, recent studies found hydrogen bonding hydrophobic interaction also play important role in the formation of complex (De Kruif and Tuinier 2001; McClements 2006). It is known that ionization of protein and ionic polysaccharide varies at different pH ranges (Figure 1), and charged molecules are deprotonated (become anionic) when environmental pH is higher than its pKa (Figure 1). When proteins and polysaccharides carry opposite charges, these charges on the back bone of protein and polysaccharide chain would drive them to attract to each other and form complex.

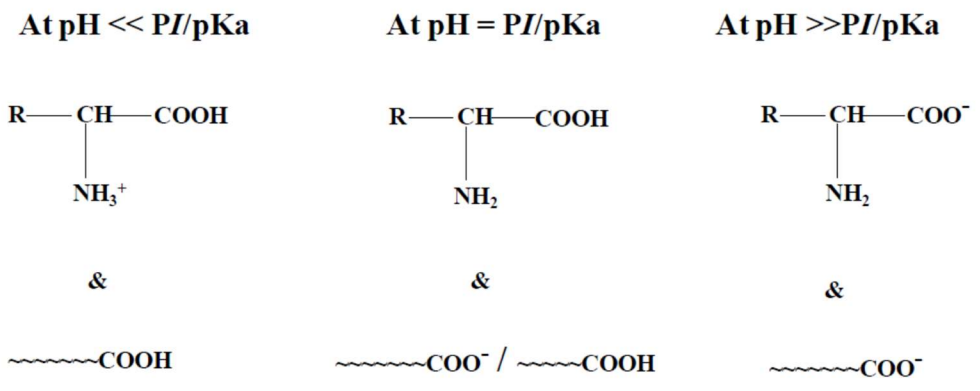

Figure 1 Chemical structures of protein and polysaccharide at different ionization modes (Ghosh and Bandyopadhyay 2012)

There has been numerous work done on studying the key factors that affect protein and polysaccharide interaction (Stone and Nickerson 2012; Benichou and others 2002). Firstly, like any other chemical reaction, the types of proteins/polysaccharides, protein to polysaccharide ratio and total biopolymer concentration can influence the degree of complexation. If protein-polysaccharide concentrations and ratios are kept constant extrinsic factors such as pH, ionic strength and temperature are the keys to control the formation of complex and modification of complex structure and properties.

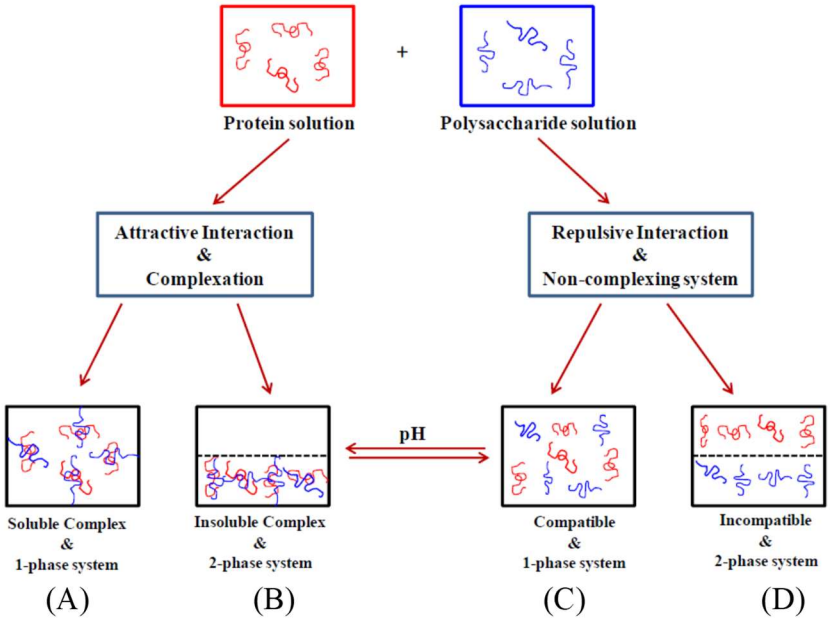

Figure 2 Mechanism of complexation at ambient temperature (Ghosh and Bandyopadhyay 2012)

Among these parameters, pH is the most important factor. When pH is lower than pI but higher than pKa, two biopolymers carry opposite charges and can agglomerate to form soluble complexes (single phase) at low biopolymer concentrations (Figure 2A). However, in some specific conditions, (for instance, the pH is extremely low, or protein concentration is high), reaction is more intensive and larger insoluble complexes are formed, resulting in associative

phase separation. The lower phase is enriched with two biopolymers (coacervates or precipitates), and the upper phase is mainly composed of solvent (Figure 2B).

On the contrary, if pH is higher than pI, the biopolymers are theoretically non-interacting and co-soluble at low concentrations (Figure 2C). Segregative phase separation (Figure 3D) occurs in concentrated biopolymer solution due to strong electrostatic repulsion (between two similarly charged bio-polymers) or very high steric exclusion between proteins and polysaccharides (Tolstoguzov, 2006). Because of incompatibility, protein and polysaccharide distribute into two different phases.

Thermal processing is another crucial factor affecting protein and polysaccharide complexation due to its effect on hydrogen bonding and hydrophobic interaction between proteins and polysaccharides as well as protein conformation (e.g., folded or unfolded) (Weinbreck and others 2004; Mitra and others 2007). Complexation under heating usually leads to larger insoluble complexes (Magnusson and Nilsson 2011). This is due to protein unfolding under heat-treatment which exposes more reactive sites (amino acids) to the solvent phase and more interaction with polysaccharides. At low polysaccharide concentration, this leads to more charge groups being neutralized (Schmitt and others 2000). The poor solubility can be modified in some extent by increasing polysaccharide concentration leading to increased surface charge and improved solubility (Figure 3A).

Contrast to ambient temperature, electrostatic interaction still happens at pH close to pI or even higher than pI under thermal processing. Some cationic reactive sites are exposed by protein unfolding under heat treatment. They can locally react with anionic polysaccharide. Compared to reaction at pH lower than pI, this reaction is milder, and more surface charge are

kept, so usually products have good solubility (O'Chiu and Vardhanabhuti 2017). Due to this feature, more concentrated soluble complex solution can be produced by this approach.

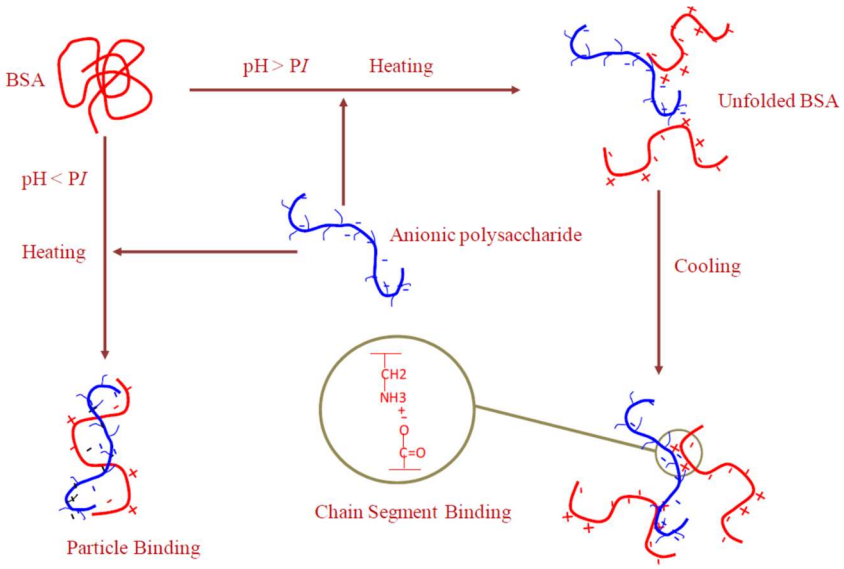

Figure 3 Mechanism of complexation under thermal process (Ghosh and Bandyopadhyay 2012)

2.1.2 Application of protein-polysaccharide complexes in food.

Due to broad potential applications, polysaccharide and protein interactions in solution have been studied during the past 30 years. Recent advancements show that this complexation could lead to: (i) improved heat stability of protein by altering protein aggregation (Ibanoglu 2005; Vardhanabhuti and Foegeding 2008; Jiménez-Castaño and others 2005) (ii) enhanced the interfacial properties of protein by forming a homogenous and thick layer around oil droplet or air bubble (Benichou and others 2002; Miquelim and others 2010; Allen and others 2008); and (iii) altered heat-induced gelation properties of protein by incorporating in protein gel network (Turgeon and Beaulieu 2001);

Our laboratory has investigated the complexation between whey protein isolate (WPI) and polysaccharides under heating and at pH > pI as well as the functional properties and potential applications of the complexes. Recent studies have shown that heated WPI and polysaccharide complexes formed at pH > pI have improved acid-induced gelation, foaming, and emulsification properties. In addition, heated WPI and polysaccharide complexes have been shown to alter protein digestion. In-vitro gastric digestion results also showed that, at certain conditions (e.g., biopolymer concentrations), intragastric gel could be formed which could potentially affect satiety and postprandial glucose response.

2.2 Emulsion-based delivery systems for lipophilic bioactive compounds

Many lipophilic bioactive compounds such as omega-3 fatty acids, carotenoids, and curcumin have been shown to have health benefits either as essential nutrients or functional foods. There are several challenges associated with incorporating them into commercial foods and beverages. Firstly, most lipophilic bioactives have poor solubility in aqueous phase, so they cannot be directly incorporated into aqueous-based foods. Secondly, certain lipophilic bioactives have melting points higher than ambient and body temperature. They are in crystalline form in food product and tend to settle and precipitate. (McClements 2012b). Thirdly, many lipophilic bioactives are susceptible to chemical degradation under food storage conditions or after ingestion. (McClements and Decker 2000; Boon and others 2010; Waraho and others 2011; Heger and others 2014). Finally, they have low bioavailability within the human gastrointestinal (GI) tract. This may occur for a number of reasons, including low bioaccessibility, poor absorption, or lack of transformation in the fluids (McClements and Xiao 2014; McClements and others 2015). So there is pressing need tor for edible delivery systems to encapsulate, protect, and release bioactive lipids.

Emulsion technology is particularly suited for the design and fabrication of delivery systems for encapsulating bioactive lipids. Compared with other delivery system, emulsions have a number of potential advantages. Firstly, they contain a nonpolar region (oil phase) where lipophilic compound can be incorporated. Secondly, emulsion is one of the most common food type. Emulsion-based system is highly compatible with many foods and beverages. Thirdly, emulsions can be created entirely from food-grade ingredients (such as water, oil, surfactants, phospholipids, proteins, and polysaccharides) using fairly simple processing operations (mixing and homogenization). Furthermore, it is also reported that, with novel composition and strategy, emulsion can enhance the chemical stability of bioactives during storage (Ezhilarasi and others 2013). Finally, many studies reported that emulsion encapsulated bioactives have higher bioaccessibility in human GI tract (Salvia-Trujillo and others 2013a; Ahmed and others 2012; Qian and others 2012a).

In this study, we focused on digestion profile and bioaccessibility of β-carotene in WPI-pectin complex stabilized emulsion in *in vitro* digestion. Therefore, digestion of protein stabilized emulsion in human GI tract should be fully discussed first.

2.2.1 Digestion of protein stabilized emulsion

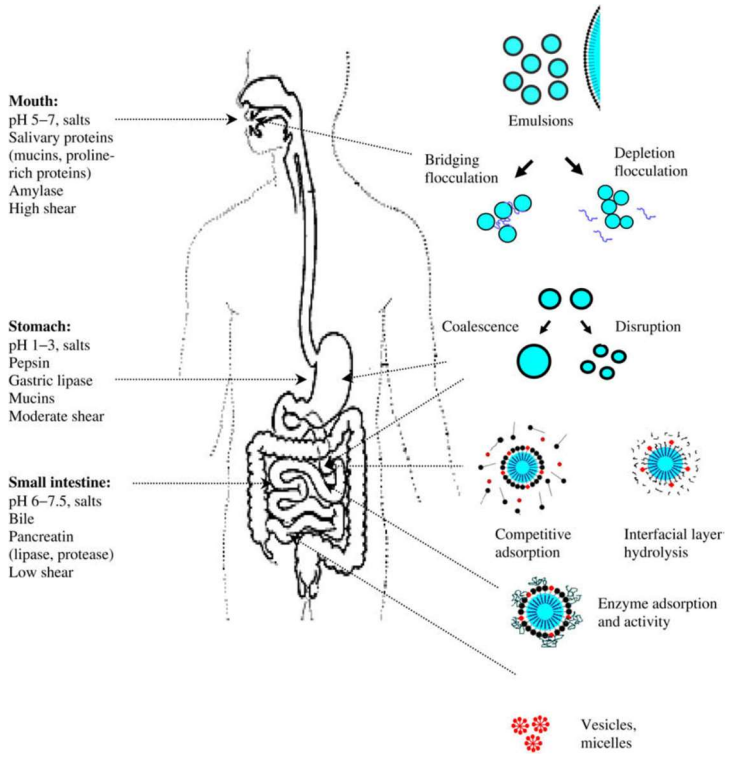

Figure 4 Digestion of emulsion in human gastrointestinal tract (McClements and Li 2010b)

Oral digestion

The first unit of the GI tract after food enters the human digestive system is the mouth. Here, food is broken down and processed both mechanically and biochemically to allow its passage through the pharynx and esophagus to the stomach (Singh and Gallier 2014). Upon ingestion, emulsion undergo significant changes because of the complex interactions in the mouth environment. Depending on the type of the food matrix, emulsion is either directly swallowed (like beverage) or chewed for a few seconds (like salad dressing) before deglutition. During chewing, changes on the structure and properties of the oil droplets depend on their initial organization within the food as well as the duration and intensity of mastication (McClements

and others 2008a). Previous study suggests that mechanical breakdown of structure could happen during chewing and leads to the release of lipid. In case of emulsion with good fluidity, key factors affecting the breakdown of the emulsions are temperature and shear force between teeth and saliva (Lemmens and others 2010). The possible impacts are primarily from the change on temperature, shear force between teeth and saliva.

Saliva is biological fluid (pH around 6.8) comprising water, proteins and minerals. Protein fraction has a complex composition, including salivary enzymes, immunoglobulins, antibacterial proteins, proline-rich proteins, lysozyme, lactoferrin, peptides and highly glycosylated mucins (Pechlivani 2015). Mucin has been found to play important role during the oral digestion of emulsions (Singh and others 2009). Being negatively charged at neutral pH, mucin can interact with positively charged emulsifier, result in bridging flocculation of oil droplets. The intensity of flocculation mainly depends on mucin concentration present in food matrix (Vingerhoeds and others 2005). In addition, recent studies found other salivary components, such as salivary salts and proline-rich proteins, also contribute to oil droplet flocculation. However, all these findings are based on *in vitro* digestion model. It is still disputable whether such flocculation really happen in human digestion. In general, there is still a relatively poor understanding of the physicochemical and structural changes that occur within the mouth when fatty foods are consumed.

Most studies dealing with the *in vitro* digestion ignore the oral digestion step under the assumption that it does not have a major impact on lipid digestion and chewing process is challenging to stimulate (Morell and others 2014). In this study, oral digestion was also not included based on reasons from both practical and theoretical aspects. Firstly, our emulsion has good fluidity and the expected time in the mouth is less than 3 s before being swallowed without

undergoing physical changes such as chewing process. Secondly, in human, saliva is very diluted with 99.5% of water. Once being mixed with 10 to 20 times the volume of emulsion-based drink, the concentration of active salivary components is too low to have an effect. Additionally, WPI-pectin complexes used in our study is negatively charged in our emulsion system and would have minimal (if any) interaction with mucin. Finally, preliminary experiment with oral digestion did not show any significant change on our emulsion system.

Gastric digestion

After the emulsion is swallowed, it rapidly slips down the esophagus into stomach, where it is surrounded by acidic gastric fluid (pH=1-3) comprising gastric enzymes (both proteolytic and lipolytic) and minerals (Ponsky and Gauderer 1981). Meanwhile, it is subjected to mechanical agitation due to stomach peristalsis. Emulsion may undergo drastic changes on both physical structure and chemical composition under these conditions.

Firstly, surface charge property could be significantly altered by a drastic decrease of pH during gastric digestion. As a result, emulsion stability would be affected, especially in case of emulsion stabilized by protein (Golding and others 2011). If the emulsion is at or near neutral pH proteins will provide negative charges on the surface. As pH drops pass the isoelectric point, proteins will be subjected to partial or full charge reversal, which leads to protein aggregating (Singh and Ye 2013). Besides pH, the ions in gastric fluid can also influence the surface charge property. The ionic strength of fasted human stomach is usually around 100 mM, mainly contributed from sodium, potassium and chloride ions (Pocock and others 2013). High ionic strength would cause shielding of electrical charges on the adsorbed emulsifier molecules, hence lower the net charge on the oil droplet surface. It should be noted that both reactions are possible to induce oil droplets flocculation and partial coalescence (McClements and Gumus 2016).

The other crucial interaction is proteolytic action of pepsin on the adsorbed protein. At 37 °C and pH 2.0, pepsin cleaves peptide bonds involving aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and other hydrophobic residues (such as leucine) at highest efficiency. The ability of pepsin to hydrolyze different proteins varies, as proteins have different tertiary conformations and possess regions with different affinities for hydrophobic and hydrophilic environments (Wieland and Bodanszky 2012). Highly disordered proteins, such as caseins, undergo rapid hydrolysis by pepsin, but proteins with highly folded conformations, such as β-lactoglobulin, show some resistance to gastric digestion in the native state (Macierzanka and others 2009). The unfolding of the native structure by heat or high pressure processing or certain chemical modifications has been shown to markedly enhance the accessibility of the specific peptide bonds needed for pepsin action (Zhang and Vardhanabhuti 2014a).

In addition, recent work suggests that adsorption of protein to the interfacial layer in an emulsion affects the rate of hydrolysis by pepsin (Sarkar and others 2009). This has been attributed to a possible change in the conformation of protein molecules upon adsorption at the oil−water interface which exposes the peptic cleavage sites for proteolysis. For example, the rate of gastric digestion of β-casein and β-lactoglobulin is enhanced in the adsorbed state in oil-in-water emulsions (Nik and others 2010).

Hydrolysis of absorbed protein causes a decrease in the net surface charge of the oil droplets (Kinsella and Melachouris 1976; Li and others 2012). In emulsions stabilized by a range of food proteins (e.g., WPI, sodium caseinate, β-lactoglobulin or β-casein), this hydrolysis can lead to flocculation and coalescence of the droplets because of insufficient electrostatic repulsions (Mao and Miao 2015). On the other hand, unabsorbed protein which refer to the protein remaining in the serum phase, also play an important role in emulsion stability. Recent

study found the presence of excess unabsorbed protein can significantly improve the resistance of oil droplets to gastric environment (Kenmogne-Domguia and others 2012). In contrast, emulsions with adsorbed layers made up of low molecular weight surfactants (e.g., phospholipids or monoacylglycerols) which are not digestible in the stomach would be acid stable and do not drastically change on structure under gastric digestion (Qian and others 2012a).

Besides pepsin, the human stomach also contains gastric lipase, which can catalyze lipolysis over a broad range of pH values (pH 2–7) (Bernbäck and others 1989). Gastric lipase binds to lipid droplet surface where it hydrolyzes the emulsified triacylglycerols (TAGs) to diacylglycerols (DAGs), monoacylglycerols (MAG), and free fatty acids (FFAs) (Gargouri and others 1986). However, a very limited degree of lipid (~ 10–30%) is hydrolyzed in the stomach (Carey and others 1983; Porter and others 2008).The impact of gastric lipase on the digestion profile of emulsions remains to be elucidated due to the unavailability of commercial gastric lipase for *in vitro* experiments.

Intestinal digestion

The partially digested emulsion that leaves the stomach is usually referred to as “chyme” (McClements and others 2008b). While chyme enters the small intestine sodium bicarbonate is being continuously secreted which results in a gradual increase in pH to 6 – 7, allowing the pancreatic enzymes to act most efficiently. Meanwhile, the ionic strength in the intestine is higher than that in the stomach (e.g., 140 mM in fasted human) (McClements and Li 2010a). Increases in pH and ionic strength result in protein charge reversal and surface charge shielding. Similar to gastric phase, these changes may lead to aggregation of protein, and flocculation and coalescence of oil droplets.

The small intestine houses many proteolytic enzymes, such as proteases and peptidases (trypsin, chymotrypsin, carboxypeptidases etc.). Trypsin and chymotrypsin are the two main proteases that catalyze the breakdown of specific peptide bonds. In protein-stabilized emulsions, because of the actions of these proteases, both the adsorbed proteins/peptides and the unadsorbed proteins/peptides are further hydrolyzed into amino acids and smaller peptides (Tavano 2013). These peptides pass into the mucus layer before undergoing subsequent cleavage to amino acids by brush border peptidases. It should be pointed out that the hydrolysis of adsorbed proteins/peptides does not occur in isolation, because bile salts and pancreatic lipases also enter the small intestine simultaneously.

Bile salts are mixtures of surface active compounds, including sodium salts of taurocholic, taurode oxycholic, taurochenodeoxycholic, glycocholic and glycodeoxycholic acids, secreted by the liver via the gall bladder. Due to their high surface activity, bile salts are very efficient at displacing adsorbed emulsifiers on oil droplet surface (Maldonado-Valderrama and others 2011). Therefore, any proteins or peptides that remain on the droplet surface are displaced by bile salts. Several *in vitro* studies have shown partial or complete displacement of protein from the droplet surface after the introduction of bile salts into the simulated intestinal fluid for emulsions formed with caseins, WPI and β-lactoferrin (Maldonado-Valderrama and others 2008). The type of adsorbed protein affects the extent of its displacement by bile salts. Whey proteins are more readily displaced than caseinates from the interface of emulsion droplets (Hur and others 2009).

Furthermore, it appears that the interactions of bile salts with proteins in water can affect their digestibility by proteases. This could apply to both the unadsorbed fractions and the adsorbed fractions of proteins in the emulsion. Gass et al. (2007) reported that bile salts

significantly increased the proteolysis of several proteins, including β-lactoglobulin, bovine serum albumin and myoglobin. For β-lactoglobulin, it was proposed that bile salts could bind into the hydrophobic pockets of the protein molecule, which might destabilize the protein structure and make additional interior domains available for protease action.

While bile salts are displacing proteins and peptide from lipid surface, lipase are adsorbing to lipid interface with facilitation of bile salts (Mackie and Macierzanka 2010). Then colipase binds to the bile-salt-rich interfacial area, and lipase is activated by forming a complex with colipase. The complex hydrolyzes the emulsified triacylglycerols (TAGs) to diacylglycerols (DAGs), monoacylglycerols (MAG), and free fatty acids (FFAs). Nevertheless, recent study suggests that, at higher concentration, bile salts have negative effect on lipid digestion. Generally, bile salts may compete with lipases for the oil–water interface and sterically hinder the adsorption of lipase. After being hydrolyzed, lipid digestion products (fatty acids, monoglycerides, cholesterol, phospholipids, and fat-soluble vitamins etc.) are solubilized by bile salt micelles and unilamellar vesicles that are composed mainly of phospholipids (van Aken 2010). These particles deliver digested lipids to the aqueous–enterocyte membrane interface and the lipid molecules are subsequently absorbed either by passive diffusion through the phospholipid bilayer of the enterocyte plasma membrane or by active transport involving specific proteins in the enterocyte brush border membrane (Fave and others 2004).

2.2.2 Bioaccessibility of β-carotene

β-Carotene is one of the major carotenoids present in fruit and vegetables that exhibits pro-vitamin A activity once ingested by humans (Johnson 2002). It is recognized that β-carotene plays an important role in the prevention of certain diseases due to its antioxidant and non-antioxidant activities (Bendich and Olson 1989). The ingestion of sufficient levels of β-carotene

has been related to a reduced risk of developing certain types of cancer, cardiovascular diseases, photosensitivity diseases, and cataracts (Mayne 1996). Therefore, there is a growing interest among researchers to enhance its bioavailability and bioaccessibility in food.

The term bioavailability has been defined as the fraction of an ingested component (or its products) that eventually ends up in the systemic circulation. Mathematically, bioavailability (F) can be defined as:

$$F = F_B \times F_T \times F_M$$

In this equation, F_B is defined as the bioaccessibility which is ingested (micro) nutrient that is incorporated into micelles, F_T is defined as the transport coefficient or the fraction of the released lipid component that is transported across the intestinal epithelium, and F_M is the fraction of the lipid component that reaches the systemic circulation without being metabolized. F_T and F_M are affected by many complex factors (McClements and Li 2010b). These properties are beyond the scope of our study.

The absorption of dietary β-carotene from nature foods involves several steps, starting with the release of carotenoids from the food matrix, followed by incorporation into lipid droplets under gastric emulsions, and then solubilization within mixed micelles under intestinal conditions (Yonekura and Nagao 2007). For this reason, β-carotene encapsulated within emulsified lipid phases tends to be more bioaccessible than β-carotene present within natural fruits and vegetables (van het Hof and others 2000). As reported, bioaccessibility is mainly related to digestion rate of lipid. L. Salvia-Trujillo and co-workers found strong positive linear relationship between bioaccessibility of β-carotene and the final FFA released (R=0.998) (Salvia-Trujillo and others 2013a). Potential mechanism is that when the lipid is digested by

gastric and pancreatic lipases it forms free fatty acids that are incorporated along with bile acids and phospholipids into mixed micelles that can solubilize and transport β-carotene to the epithelium cells (Tyssandier and others 2001). So, in general, any factor that affects digestion of lipid would have effect on bioaccessibility of β-carotene.

2.2.3 Digestibility and releasing of emulsified lipid

As we talked in last section, bioaccessibility of β-carotene is mainly based on digestion and releasing of lipid in intestinal phase. A number of structural design approaches of emulsions have been developed to enhance bioaccessibility by controlling lipid digestion and release. Following, we briefly review crucial factors that affect digestion of lipid and how they were utilized to control digestibility of lipid, and, further, bioaccessibility of oil.

Generally saying, lipid digestion is an interfacial phenomenon that involves adsorption of lipase molecules to lipid droplet surfaces so that the enzyme can come into close proximity with its substrate (usually triacylglycerols). So, any factor that affects the binding of lipase to the oil–water interface, such as the surface area of the interface, the molecular structure of lipid molecules or the nature of the emulsifier, would be expected to have an impact on the rate and extent of lipid digestion.

Formulate Oil composition and content

Different types of lipids are used to prepare emulsion-based foods. Previous studies have shown that oil type has a major impact on lipid digestion and release. For example, long chain triglycerides (LCT) are known to be digested at a slower rate than medium chain triglycerides (MCT), which has been attributed to differences in dispersibility of the FFAs in water during digestion (Porter and others 2007; Pouton and Porter 2008). The medium chain FFAs produced

during digestion of MCT oils are able to migrate rapidly into the surrounding aqueous phase and so they do not inhibit the interfacial lipase reaction. Oppositely, the long chain FFAs produced by LCT oils tend to accumulate at the oil-water interface and inhibit lipase activity until they are removed by being solubilized in micelles or precipitated by calcium ions (Jandacek and others 1987). The rate and extent of lipid digestion in the emulsions were clearly higher for MCT (max=100%) than for LCT (corn oil, max=70%) (Porter and others 2007).

However, the effect is opposite for β-carotene. Qian et al. (2012a) found that whereas bioaccessibility decreased in the order LCT >> MCT > orange oil. She attributes this phenomenon to lack of mixed micelles were formed to solubilize β-carotene in orange oil, mixed micelles were too small to solubilize β-carotene, nevertheless the long chain fatty acid has higher solubilization capacity. This theory was confirmed by Salvia-Trujillo's research, she found with LCT content increasing in oil receipt, there was a progressive enhancement in β-carotene bioaccessibility (Salvia-Trujillo and others 2013b). Besides, her research also suggested higher carrier oil concentration benefited bioaccessibility of β-carotene.

Lower droplet size (enlarge surface area)

The specific surface area of the lipids in an emulsion is given by the following expression:

$$A_N = \frac{6\phi}{D_{43}}$$

where, A_N is the surface area of lipids exposed to the aqueous phase per unit volume of emulsion, D_{43} is the mean diameter over volume, and ϕ is the disperse phase volume fraction (McClements and Li 2010b). One would therefore expect the lipid digestion rate to increase with

decreasing droplet size, since this would lead to an increase in the surface area of lipid exposed to the lipase. Studies have reported that lipid digestion rate increases with decreasing droplet size, when the digestion rate is expressed as FFAs released per unit time. (Armand et al. 1992; Borel et al. 1994; Bauer et al. 2005; Lundin and Golding 2009). For β-carotene delivery, consistent results were found by Salvia-Trujillo. Bioaccessibility increased with initial droplet size decreasing, large (D_{43}= 23 μm, bioaccessibility=35%) < medium (D_{43} = 0.4 μm, bioaccessibility=50%) < small (D_{43} = 0.2 μm, bioaccessibility=65%) (Salvia-Trujillo and others 2013c). Consequently, the size of droplets reaching the small intestine may be quite different from those ingested. Therefore, it may be important to control both initial droplet size and physical stability of lipid droplets within the GI tract in order to control the rate of free fatty acid release.

Emulsifier type and concentration

A variety of emulsifiers are being used in food industry nowadays including small molecular weight surfactants, biopolymers and phospholipids (Whitehurst 2008; McClements 2015). Many papers have been published on investigation of the impact of initial emulsifier type on the lipid digestion process.

Chu and co-workers found that the rate of lipolysis of lipid droplets could be inhibited by coating them with galactolipids with large hydrophilic head-groups that retard adsorption of bile salts and lipase through steric hindrance (Chu and others 2009). Reis and coworkers found that the rate of lipid digestion was lower for droplets initially coated by monoglycerides than those coated by proteins or phospholipids(Reis and others 2008). Mun previously reported that the resistance of lipid droplets initially coated by different types of emulsifier to lipid digestion decreased in the following order: nonionic surfactant (Tween 20) > phospholipids (lecithin) >

protein (caseinate or WPI) (Mun and others 2007). Thus, proteins (especially milk proteins) are promising in designing deliver system.

Increasing the concentration of surfactant in emulsions is widely used as an approach to decrease the emulsion droplet size during homogenization and enhance the stability of emulsion during storage (Araiza-Calahorra and others 2018). More importantly, higher surfactant concentration creates thicker interfacial layer that maintains the droplet size and integrity of interfacial layer during digestion (Mun and others 2007). Consequently, the lipid digestion could be facilitated. Nevertheless, some studies suggest that overly increasing the surfactant concentration can result in barrier effect, which means the thick emulsifier layer is difficult to be displaced by bile salts and also hinders the adsorption of lipase, leading to a decrease in the amount of FFA released. Nevertheless, it should be noted that the interfacial composition may change appreciably as lipid droplets pass through the GI tract due to digestion and competitive adsorption phenomena, e.g., with bile salts, phospholipids or FFAs (McClements and others 2008a; Dickinson 2013). Hence, the characteristics of the droplet interfaces in the small intestine might be considerably different from those of the ingested emulsion.

2.2.4 Effect of polysaccharide on emulsion digestion

Polysaccharides can interfere with the digestion process of emulsion in variety of ways. Firstly, they may interfere with the physical property of initial emulsion and stability of emulsion during digestion. It was reported that emulsification process of bulk ingested fats could be altered by polysaccharide. With presence of chitosan, droplet size surface charge and viscosity could was significantly changed (Armand and others 1999). Changes on these features could further influence digestion property of emulsion in GI tract. For instance, because of increases on viscosity, the magnitude of the shear forces operating on the chyme in stomach would be

different. Also, recent studies suggest presence of polysaccharide are beneficial for stability of protein stabilized emulsion in GI tract. Zhang and co-workers showed that with pectin, lactoferrin stabilized emulsion had smaller droplet size after intestinal digestion (Zhang and others 2015). Chang reported fucoidan benefited stability of emulsions, which are separately stabilized by caseinate and whey protein, in both gastric phase and intestinal phase (Chang and McClements 2016b). Xu found that by forming conjugate with pectin, droplet size of whey protein stabilized emulsion could be significantly reduced in GI tract (Xu and others 2014b). They attributed this phenomenon to strong surface charge and steric effect of adsorbed polysaccharide.

Secondly, polysaccharides affect digestion of emulsified lipid. Polysaccharide can bind with bile salts and phospholipids in the small intestine. This alters the digestion process by reducing the amount of surface active compounds available to stabilize the fat droplets or by retarding the transport of digested lipids from the droplets to the intestinal wall via mixed micelles (Dongowski 1997; Thongngam and McClements 2005). Besides, they can directly react with the lipase and/or co-lipase, thereby reducing its enzyme activity (Han and others 1999). Also, adsorbed polysaccharide on emulsified lipid can perform as a protective coating which hinders bile salt adsorbing to the droplet and prevents lipase/co-lipase from getting access to lipids inside the droplets. In addition to impact on lipid digestion, literature suggests protein digestion profile can be altered by presence of polysaccharide. The addition of pectin in heated β-lactoglobulin decreased the degradation rate of protein, probably induced by local protein-pectin interactions, which decreased the accessibility of cleavage sites to pepsin (Peyron and others 2006) . Another study using kiwi protein also showed that addition of apple fruit pectin was able to protect kiwi allergen from pepsin digestion (Polovic and others 2007).

Nowadays, encapsulation and delivery of nutritional compounds become the hottest field in food science academia. Many deliver systems have been developed to control lipid digestibility and enhance bioaccessibility of lipophilic bioactive compounds. However, to best knowledge the author, rare study has been done with WPI-pectin complex stabilized emulsion.

CHAPTER 3 MANUSCRIPT

3.1 Introduction

β-carotene plays an important role in human health and lowering risks of various diseases such as cancer, heart disease and colorectal adenomas. These benefits have raised the interests of the food industry in incorporating β-carotene in commercial food products. However, due to the nature of β-carotene (e.g., low water solubility, high melting point, poor chemical stability, and limited bioaccessibility), there are many challenges associated with their delivery and applications in foods. Studies suggest that oil-in-water emulsion would be one of the most suitable type of food for incorporating β-carotene. Emulsion system not only enhances β-carotene chemical stability during storage (Qian and others 2012b), but also improves its bioaccessibility in human digestion tract compared with natural food matrix (Zhang and others 2016a).

The term ‘‘bioaccessibility’’ has been defined as the fraction of an ingested compound that is solubilized within intestinal fluids in a form that is available for absorption (Benito & Miller, 1998). The bioaccessibility of β-carotene can be affected by many factors including the nature of the original food matrix, the type of food processing steps involved, and the concentration, structure and conformation of the β-carotene and lipids consumed (Castenmiller and West 1998). In emulsion, β-carotene bioaccessibility is mainly related to digestion of lipid. Before absorption, β-carotene needs to be solubilized in micelles formed from lipid digestion products and bile salts before being transferred within the micelles to intestinal membrane. Strong positive linear relationship between bioaccessibility of β-carotene and the final free fatty acid (FFA) released (R=0.998) was found by Salvia-Trujillo and others (2013a). Thus, there is a

great potential to modify bioaccessibility of β-carotene through controlling digestion of emulsified lipids in human gastrointestinal (GI) tract.

Many attempts have been made on development of emulsion system to achieve the goal of controlling emulsified lipid digestion and release. Besides oil composition, oil-water interfacial structure and oil droplet size are believed to be the most crucial factors to control lipid digestion (Rao and others 2013; McClements and others 2008b). Researcher successfully facilitated lipolysis by lowering oil-droplets size into nanometer level (Ahmed and others 2012). Also, recent study found that milk protein (whey protein) stabilized emulsion has better lipid releasing property compared with emulsions stabilized by nonionic surfactant (Tween 80) and emulsion stabilized by phospholipids (lecithin) (Mun and others 2007). However, it should be noted that lipid droplet size and structure of oil-water interfacial layer may change appreciably as emulsions go through the GI tract, due to droplet disruption, coalescence, flocculation or enzymic digestion (McClements and others 2008a; Sarkar and others 2009). Consequently, emulsion reaching the small intestine may be quite different from those ingested. Therefore, it is important to control emulsion stability within the GI tract in order to control free fatty acid release.

Many studies have reported that pectin could benefit the stability of emulsion during digestion (Verrijssen and others 2014a; Verrijssen and others 2016; Xu and others 2012). It may due to the fact that pectin is not digestible by human and has excellent charge property. Nevertheless, recently literatures suggested that pectin also inhibits digestion of oil lipid. The mechanisms are i) pectin hinders lipase adsorbing to oil droplet surface (Verkempinck and others 2018c). 2) pectin binds with bile salts, thus lowering availability of bile salt to form micelle (Vahouny and others 1980).

Our laboratory recently developed a whey protein-pectin complex formed at neutral pH under heat treatment. Previous work showed formation of complex can significantly enhance the emulsifiability of whey protein. In this study, we further investigated the digestion properties of emulsions stabilized by whey protein-pectin complex in *in vitro* digestion model. Besides, the potential use of these complexes to develop a β-carotene deliver system was investigated.

3.2 Materials and Method

3.2.1 Materials

Whey protein isolate was the gift from Davisco Foods International (BiPro, Le Sueur, MN, USA). As stated by the manufacturer, the WPI was constituted of 95.4% total solid, 93.1% protein and 0.1% ash. Low methoxyl pectin (LM-12), was kindly donated by CPKelco (Atlanta, GA, USA). It is derived from citrus peels, and has a degree of methyl esterification of 40%. Corn oil was purchased from a local Walmart Store. β-carotene, Pepsin from porcine gastric mucosa (≥ 400 units/mg), porcine lipase (100–500 units/mg), porcine pancreatin (8 × USP specifications) and porcine bile extract were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). All other chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were analytical grade or higher.

3.2.2 Preparation of β-carotene emulsion

Stock solution

WPI stock solution (10% w/w) was prepared by slowly dissolving the protein in distilled deionized (DDI) water (Millipore water,18.2 MΩ) with continuous stirring for 2 h at room temperature (25 ℃). Pectin stock solution (2% w/w, pH 7) was prepared by hydrating pectin at 65 ℃ for 2 h under continuous stirring before cooling, pH adjustment, and final weight

adjustment. The stock solutions were kept in the refrigerator (4 ℃) overnight for complete hydration. On the next day, these stock solutions were warmed to room temperature prior to next step.

Emulsifier solution

WPI and pectin stock solutions were warmed up to room temperature (25 ± 1 ℃) for at least 1h. The two biopolymer solutions and water were mixed at an appropriate amount and pH was adjusted to 7.0 ± 0.02. Water was added such that the final solutions contained 3% protein and 0, 0.3 or 0.6% pectin which corresponded to 0, 0.1 and 0.2 pectin to WPI. Mixtures were stirred for at least 30 min at room temperature. Then for heated group, samples were heated at 85 ºC for 30 min. The total of 6 treatments were unheated WPI (WPI), heated WPI (HWPI), unheated mixed WPI and pectin at 0.1 or 0.2 pectin to WPI weight ratio (MIX 0.1 and MIX 0.2), and heated mixed WPI and pectin at 0.1 or 0.2 pectin to WPI weight ratio (HMIX 0.1 and HMIX 0.2).

Emulsification

Oil phase was prepared by dispersing β-carotene (0.345%, w/w) in corn oil by sonicating (1 min) and mild heating at 50 ºC for 5 min to ensure complete dissolution. Aqueous phase was prepared by diluting the solutions with DDI water, (refer Appendix I for detailed formula). Primary emulsion was prepared by mixing oil phase (1.5 g) and emulsifier solution (28.5 g) with ULTRA-TURRAX high speed blender (IKA, Staufen im Breisgau, Germany) at 12,000 rpm for 1 min. The final emulsion was obtained by further sonicating primary emulsion at 40% amplitude for 3 min using a Vibra-Cell Ultrasonic Liquid Processors (Sonics, Newtown, CT) under ice water bath.

3.2.3 *in vitro* digestion

Emulsions were subjected to a static consensus *in vitro* digestion method consisting of gastric phase and small intestinal phase. Stimulated gastric fluid (SGF) and stimulated intestinal fluid (SIF) were prepared based on a standard formula (Minekus and others 2014). See appendix for detailed recipe.

Stomach phase

SGF consisted of 6.9 mmol L^{-1} of KCL, 0.9 mmol L^{-1} of KH_2PO_4, 26 mmol L^{-1} of $NaHCO_3$, 46.2 mmol L^{-1} of NaCl, 0.1 mmol L^{-1} of $MgCl_2(H_2O)_6$, 0.5 mmol L^{-1} of $(NH_4)_2CO_3$. Pepsin solution (0.0625 g/mL) was made by dissolving porcine pepsin in SGF electrolyte stock solution. Then pepsin solution and SGF electrolyte stock solution were kept in shaking water at 37 ºC for warming up at least 30 min. 10 mL emulsion sample was taken, then mixed with 1.6 mL pepsin solution, 6.4 mL SGF electrolyte stock solution, 50 μL of 0.3 M $CaCl_2$, 0.4 mL of 1 M HCl and 1.55 mL of water. pH need to be verified at 3 with 1N and 0.1N HCl. Then mixture was put into shaking water bath (37 ºC 95 rpm) (Thermal SW23) immediately and subject to 120 min gastric digestion.

Intestinal phase

After the gastric digestion, the pH of the samples were immediately adjusted to 7.00 ± 0.2 with 0.1 M NaOH solution and volume of NaOH consumed was recorded. Afterwards, cheyme was gently well shaked, then 20 mL was taken and was mixed with 12.5 mL of SIF electrolyte stock solution and 3.5 mL fresh bile (53.6 mg/ml), 2.5 ml enzyme solution (24 mg/ml lipase, 5 mg/ml pancreatin), and 1.5 mL $CaCl_2$ (27.745 mg/ml). SIF electrolyte stock solution comprised 6.8 mmol L^{-1} of KCL, 0.8 mmol L^{-1} of KH_2PO_4, 85 mmol L^{-1} of $NaHCO_3$, 38.4 mmol L^{-1} of

NaCl, 0.33 mmol L^{-1} of $MgCl_2(H_2O)_6$. Enzyme and fresh bile were made with 0.05 M phosphate buffer. It should be noted that, pH of all solutions must be at 7 before mixing. Because once mixing all ingredients, enzymatic reaction would occur very fast. Samples were moved to shaking water bath for 2 h digestion upon mixing all ingredients.

3.2.4 ζ-Potential Measurements

Surface charge was measured by the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom) equipped with 633 nm laser and 173° detection optics. Samples were diluted with phosphate buffer (0.05 M, pH7), SGF or SIF to a final protein concentration of 0.002% w/w for measurement. Dilution with according solution is indispensable due to the fact that concentrated large droplets may cause multiple scattering effects, and change of iron environment may result in variation on surface charge property. Both can bring nonnegligible bias into the measurement. For each sample, three measurements were conducted with at least 12 runs and each run lasted for 10 s. Surface charge was reported as the average ζ-potential.

3.2.5 Measurements of Oil droplet diameter

The particle size and size distribution were measured before and during the *in vitro* digestion process. The particle size distribution was measured using static light scattering (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted in 400 ml DDI water and stirred in the dispersion unit with a speed of 1250 rpm. The particle size was reported as surface-weighted mean diameter (D_{43}).

3.2.6 Measurement of free fatty acid (FFA) release

During intestinal digestion, pH was maintained at 7.0 by the addition of 0.25 M NaOH. The amount of NaOH added over time (5, 10, 15, 20, 30, 40, 60, 90,120 min) was recorded. The percentage of free fatty acids released was calculated from the number of moles of NaOH required to neutralize the FFA using the following formula:

$$\%\text{FFA} = \frac{V_{NaOH} \times m_{NaOH} \times M_{Lipid}}{2 \times Moles_{Lipid}} \times 100$$

Here V_{NaOH} is the volume of NaOH required to neutralize the FFAs produced (in mL), m_{NaOH} is the molarity of the NaOH solution (0.25 M), W_{Lipid} is the total weight of lipid initially present in the reaction vessel (0.5 g), and $Moles_{Lipid}$ is the molecular weight of corn oil (800 g/mol) (Espinal-Ruiz and others 2016). Percent FFA release is a direct index reflecting digestibility and digestion profile of lipid.

3.2.7 Confocal laser scanning microscopy (CLSM)

Samples was mixed with Nile Red solution (0.1% w/w) at 4:1 sample to Nile Red weight ratio before vertexing at 500 rpm for 1 min. The mixtures were kept out of light for 3 min for the lipid to be completely dyed. Approximately 20 μL of the dyed samples was placed into a normal slide, covered with a 0.17 mm coverslip. The structure was observed in situ by a Zeiss LSM 510 META confocal laser scanning microscope (Cal Zeiss, Jena, Germany) with 63× water immersion objectives. A visible light laser was used with an excitation wavelength of 545 nm. HyD2 detector was used and wavelength of detector was set with range of 565-626 nm. Digital image files were acquired in 1024 pixels × 1024 pixels.

3.2.8 Determination of β-carotene release

The bioaccessibility of β-carotene was determined after each sample had been subjected to the gastric and intestinal digestions as described previously (Wang and others 2016). Raw digesta samples were collected and centrifuged at 11617 g, 4 °C for 30 min. In sample from gastric phase, the released β-carotene was assumed to be distributed in the supernatant, and the aggregate layer contains emulsified oil droplets, part of proteins and pectins. For intestinal sample, the supernatant was assumed to be the "micelle" fraction, in which the β-carotene was solubilized. After centrifugation, the supernatant was collected and 50 ml extraction solvent, containing hexane, acetone and ethanol (50:25:25) was added. The mixtures were centrifuged at 7435 g for 10min and the supernatant phase was collected, and bulk oil was removed if applicable. The absorbance at 450 nm was measured using a UV-Visible spectrophotometer (Varian 50 Bio). The amount of β-carotene was determined using a standard curve established with β-carotene standard solutions dissolved in hexane. The absorbance at 450 nm was calibrated using control emulsions without β-carotene that were prepared and digested at identical conditions to those with β-carotene, before determining the amount of released β-carotene, as presented previously.

The bioaccessibility of β-carotene was calculated using the following equation.

$$\%\ \text{Bioaccessibility} = 100\ \times \frac{W_{Micelle}}{W_{total}}$$

where, $W_{micelle}$ and W_{total} are the mass of β-carotene in the mixed micelle phase after the simulated digestion, and total β-carotene originally added respectively (Liu and others 2012).

3.2.9 Statistical analysis

The statistical software program SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) was used to perform all statistical analysis. Results are reported as means and standard deviations. Significant differences in particle electrical charge and mean sizes were conducted by one-way ANOVA and the Tukey's Studentized Range Post-hoc test with a 95% level of significance ($P < 0.05$). All particle charge and size measurements were performed in three duplicates at least.

Nonlinear regression was used to model the % FFA release data obtained through the kinetic study for lipid digestion. It was developed by Verkempinck through model discrimination (2018b). It is a fractional conversion model with mathematical equation:

$$C = C_f \times (1 - e^{(-kt)})$$

Hereby C (%) is the percentage of FFA released at time point t. C_f (%) is the final FFA concentration that can be obtained with the imposed conditions; k (min^{-1}) is the reaction rate constant of the studied process and t (min) is time in the simulated intestinal phase.

3.3 Results and Discussion

3.3.1 Initial emulsion

After emulsification, mean droplets size (D_{43}) and ζ-potential of fresh emulsions were measured with laser diffraction system and laser Doppler micro-electrophoresis system, respectively. Microstructures were imaged by confocal microscope. As shown in Figure 1, all emulsions shared a similar initial structure with small and homogenous oil droplets dispersed in the continuous phase. Mean droplet diameter results confirmed the microstructural results showing the range of D_{43} from 0.739 to 0.826 μm with no significant difference among samples

(see Appendix II for detailed data). Small sizes and the absence of flocculation indicated the formation of good emulsions which could be due to charge stabilization from whey protein where pH is further from pI. As shown in Table 1, ζ-potentials of these emulsions were highly negative with no significant difference among samples ($p > 0.05$). All samples were negatively charged because at pH 7 both whey protein (pI ≈5.2) and pectin ($pK_a < 3$) are anionic. As a conclusion, all six samples showed similar initial physiochemical properties.

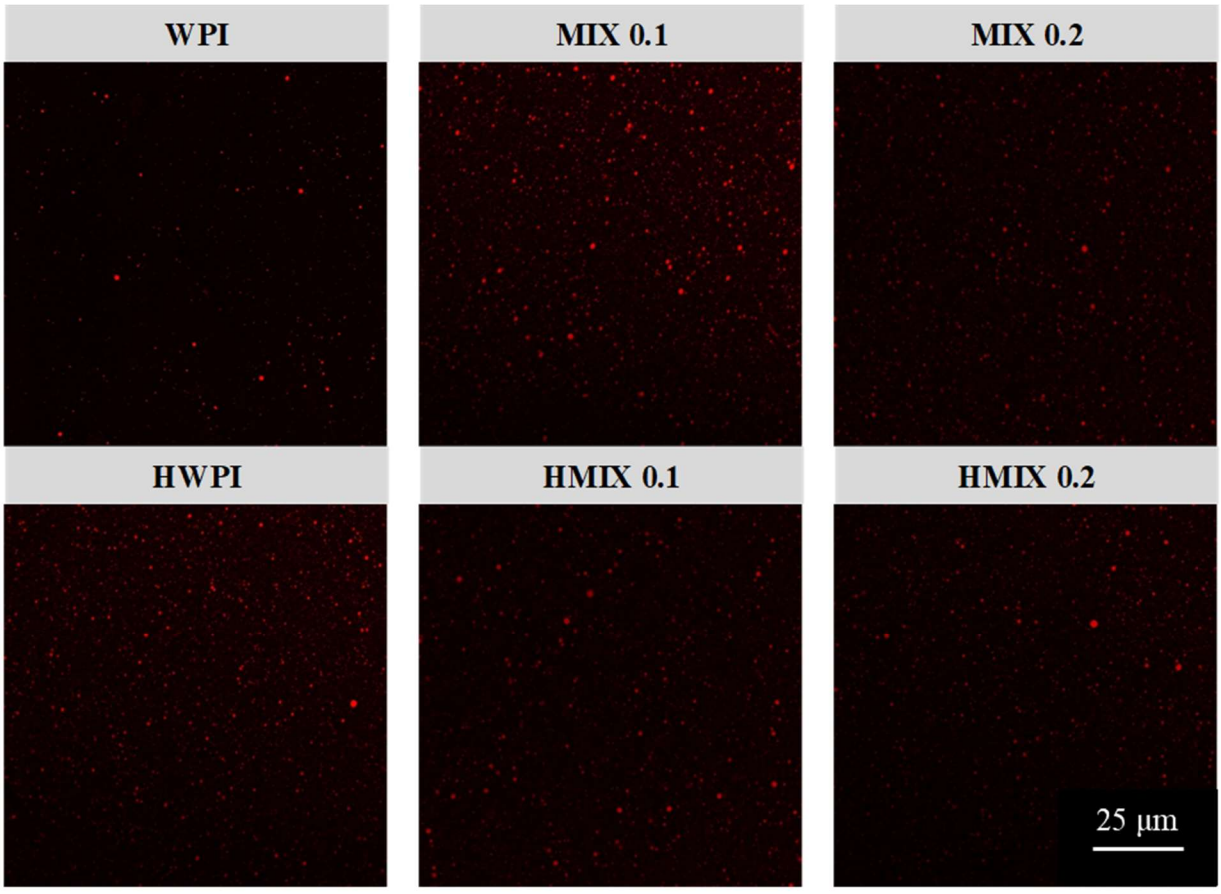

*Figure 5 Microstructure of initial emulsions stabilized by different emulsifiers: unheated WPI (**WPI**), unheated WPI-pectin mixture whose pectin concentration is 0.1 wt.% or 0.2 wt.% pectin (**MIX0.1** and **MIX 0.2**), heated WPI (**HWPI**) and heated WPI-pectin complex whose pectin concentration is 0.1 wt.% or 0.2 wt.% (**HMIX 0.1** and **HMIX 0.2**).*

3.3.2 Gastric digestion

3.3.2.1 ζ-potential

Freshly made emulsions were subjected to 2 hours of *in vitro* gastric digestion. ζ-potentials of the samples at 0 min (e.g, the time point right after mixing the sample with SGF and enzymes) and 120 min (e.g, the end of gastric digestion) were shown in Figure 6. Once emulsion was mixed with SGF, surface charge of all samples drastically changed from negative to positive due to the change of pH from 7 to 3. In addition, the ionic strength rose to 100 mM in SGF (Minekus and others 2014; Estévez-Santiago and others 2016). The change in charge property was expected for WPI and HWPI with the ζ-potentials of 17.4 ±1.33 mV and 19.1±1.35 mV, respectively. When pectin was present surface charge was less positive (e.g., lower ζ-potential) compared to sample without pectin. At SGF pH, protein had a net positive charge and pectin was negatively charged. Protein could react with pectin via electrostatic attractive force which caused charge neutralization and resulted in lower positive charge potential especially at higher pectin concentration (Zhang and Vardhanabhuti 2014b). Interestingly, MIX samples were less positively charged compared to HMIX samples. Our previous work showed that when WPI and pectin were heated together at pH 7, interactions could occur between the positively charged patches on the protein and negatively charged pectin (Zhang and others 2012). Emulsification could lead to more absorption of excess pectin at the interface, but part of pectin could already attach to protein during heating. Thus, one potential reason is that there was less excess pectin in HMIX, resulting in less charge neutralization. Another potential reason is that MIX emulsions were more unstable and became coalesce which could lead to drastic change in the surface charge property.

No change in ζ-potential was observed in WPI and heated WPI emulsions after 120 min digestion. Similar finding has been reported with β-lactoglobulin stabilized emulsion (Li and others 2013). For MIX emulsions, ζ-potential significantly decreased at the end of gastric digestion, indicating less positively charged. During digestion proteins were hydrolyzed by pepsin, thus both negatively charged and positively charged patches were exposed to solvent. This allowed more reactive sites for anionic pectin molecules. The positive charges were neutralized by pectin, so the net surface charge was reduced (Xu and others 2014a). For HMIX emulsions, the ζ-potentials at 120 min were slightly lower compared to at 0 min; however, the differences were not significant ($p > 0.05$). In contrast to MIX, HMIX samples maintained higher positive charge.

In summary, drastic changes on ζ-potential in gastric digestion mainly can be attributed to three effects: extreme acidic environment with high ionic strength, proteolysis of whey proteins and charge neutralization effect of pectin. Importantly, changes of surface charge could lead to profound effect on emulsion structure which will be fully discussed in next section.

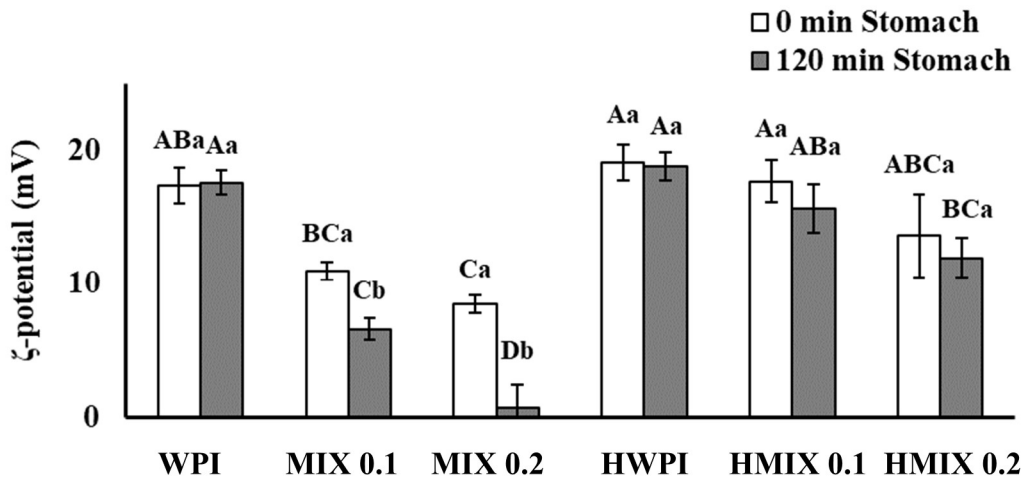

Figure 6 ζ-potential of emulsions stabilized by WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin), heated WPI and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) at the 0 min (White) and 120 min (Gray) of gastric digestion.
Different capital letters indicate significant (P<0.05) difference between different samples at the same time point. Different lowercase letters indicate significant (P<0.05) difference between same sample at the different time points.

3.3.2.2 Emulsion Structure

Weighted average mean droplet diameter (D_{43}) , size distribution, and microstructure were determined to characterize the change of emulsion structural properties during digestion. It should be noted that flocculation and coalescence of oil droplets occurred in the gastric phase, so D_{43} may represent flocculated oil droplet clusters instead of individual droplets. Furthermore, D_{43} is a mean droplet size weighted by volume, so if there are some extremely large particles, D_{43} would be heavily leveraged (Golding and others 2011). Therefore, to have a more direct observation, microstructure was imaged with confocal microscope.

As shown in Figure 7 and 8, once emulsions were mixed with SGF the mean droplet sizes drastically increased with broad size distribution (initial emulsion size ≈ 0.7 μm), indicating

droplets coalescence and/or irreversible flocculation. Significant differences ($p < 0.05$) in D_{43} among samples were observed. Overall, emulsions stabilized by WPI showed much larger increase in droplet sizes and the presence of higher pectin content also led to larger D_{43}. After 2 h digestion, D_{43} of WPI and MIX 0.1 significantly increased from time 0 while no significant changes in D_{43} were observed in other samples. The confocal microscopic images (Figure 9), however, provided a much clearer understanding of changes during gastric digestion. For WPI emulsion, oil droplets profusely coalesced with few flocculated clusters of droplets. After 2 h, emulsion completely lost its initial structure and the oil phase appeared as one connected mass. It is known that whey proteins aggregate at pI and denature at low pH condition (McClements 2004). Aggregating may happen among proteins adsorbed on the same oil droplet and those on different droplets, causing bridging flocculation of oil droplets as shown . Coalescence then occurs due to the lack of electrostatic repulsion, especially when whey proteins went through pH change from neutral to gastric pH (Chang and McClements 2016a). In contrast, compared to WPI sample, confocal images of HWPI showed much lower degree of flocculation and coalescence both at time 0 and after 2 h digestion. Microstructural results correspond to the previous droplet size results. It has been suggested that soluble whey protein aggregates formed during heat treatment have better emulsifying ability and stability under acidic condition (Sandra and others 2008). This is mainly attributed to the aggregates being able to form thicker layer at the oil-water interface which results in the higher resistance to coalescence.

In the emulsions contained pectin flocculation of oil droplets were observed. Flocculation was much more extensive when the emulsions were stabilized by protein-pectin mixture (e.g., MIX and HMIX. These microstructural results corresponded to the mean droplet size results. At the same amount of pectin, MIX samples had larger D_{43} compared to HMIX.

Among MIX or HMIX samples, those containing higher pectin content (0.2%) showed larger D_{43}. The observed flocculation was likely due to bridging flocculation caused by electrostatic interactions between anionic pectin and positively charged whey protein under gastric pH (Chang and McClements 2016a). After 2 h gastric digestion, substantial coalescence occurred in emulsions stabilized by MIX similar to the WPI sample though MIX samples showed some flocculated droplets that did not undergo coalescence. Interestingly, much smaller degree of coalescence was observed in both HMIX 0.1 and HMIX 0.2 and D_{43} of HMIX emulsions did not change after digestion. As mentioned above, flocculation in these samples was not as severe as the MIX emulsions because HMIX samples could form thicker layer at the oil droplet surface. In addition, higher positively charged observed in HMIX samples could prevent droplets flocculation and coalescence.

It should be noted that D_{43} results may not be consistent with what was shown from microscopic images, especially for the samples with flocculated oil droplets. In size determination, samples were diluted before measurement while microstructural analysis was conducted on undiluted emulsions (Chanamai and McClements 2001). Sample dilution could weaken attraction between flocculated droplets via depletion flocculation and bridging interactions (e.g., by promoting desorption of adsorbed polymers from droplet surfaces (Salvia-Trujillo and others 2013c). To confirm, we also observed diluted sample under confocal microscope and found that flocs were partly dissociated by dilution (data not shown). As a consequence, mean droplet sizes were usually smaller than the actual sizes. In summary, the major changes of emulsions in gastric digestion were flocculation caused by pectin as well as coalescence caused by drastic pH change and proteolysis.

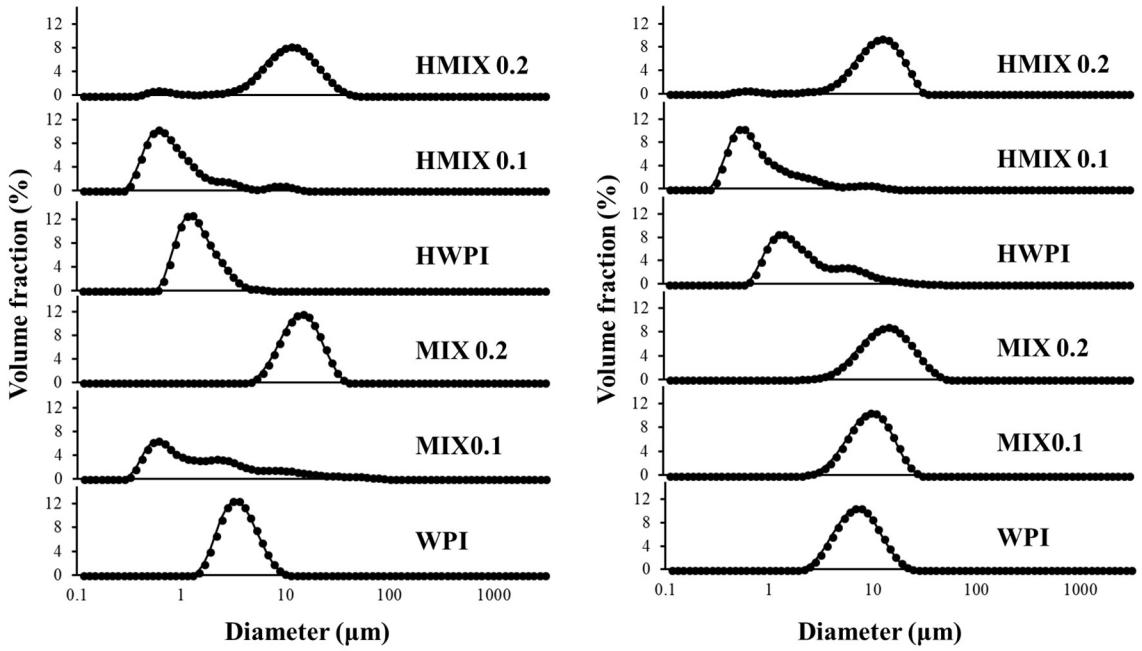

Figure 7 Particle size distribution of emulsions stabilized by WPI, heated WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin) and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) at the 0 min (A) and 120 min (B) of gastric digestion.

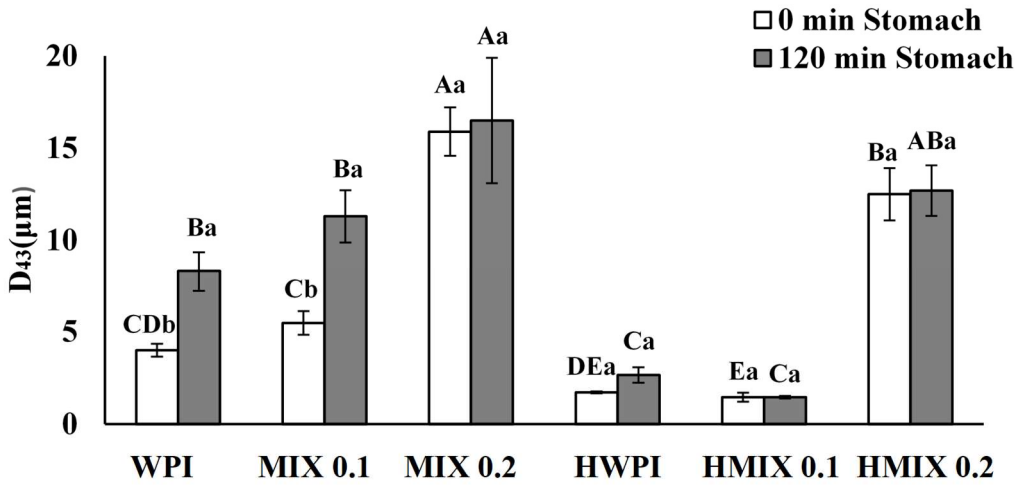

Figure 8 Mean particle size (D_{43}) of emulsions stabilized by WPI, heated WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin) and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) at the 0 min (White) and 120 min (Gray) of gastric digestion.

Different capital letters indicate significant ($P<0.05$) difference between different samples at the same time point. Different lowercase letters indicate significant ($P<0.05$) difference between same sample at the different time points.

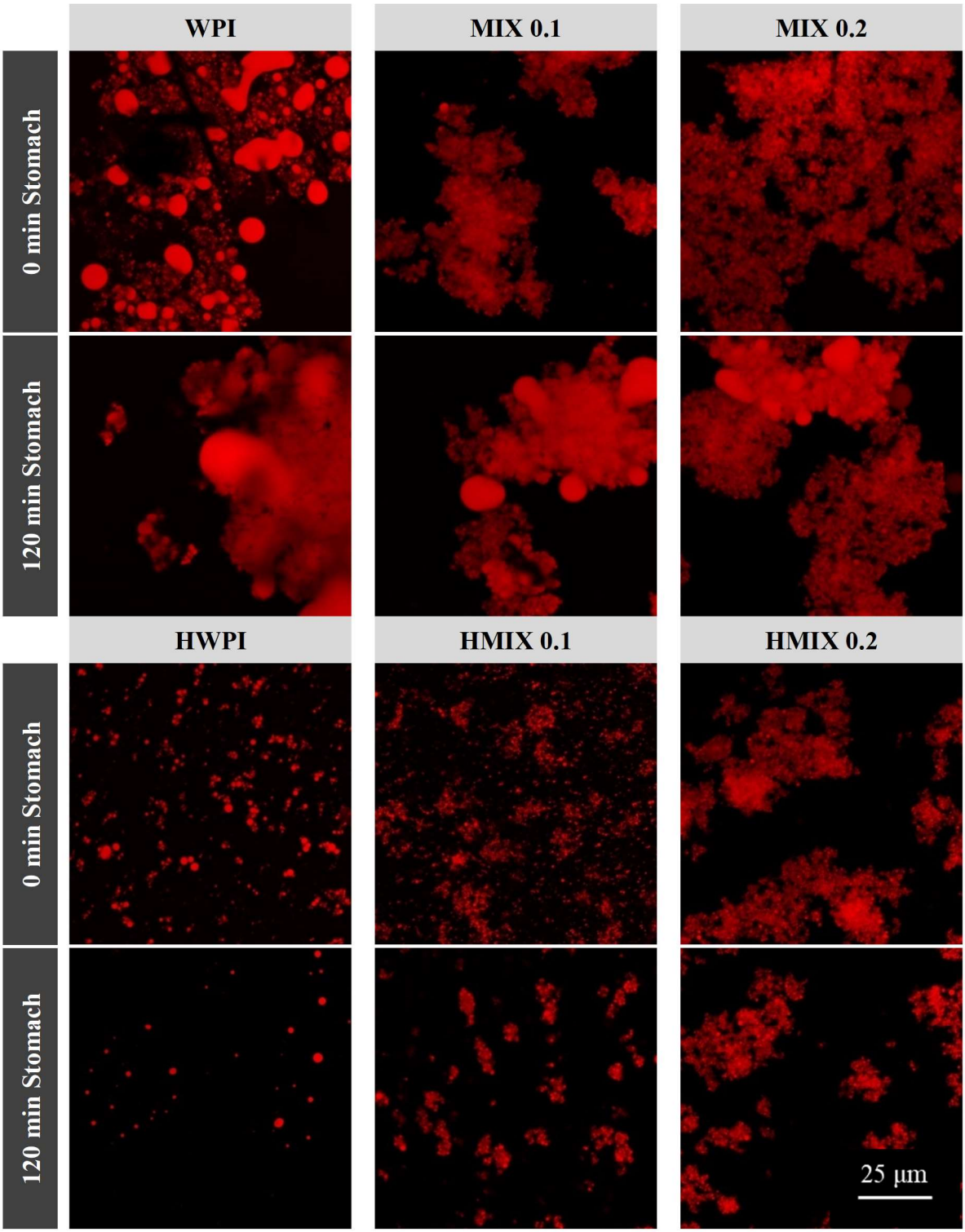

Figure 9 Microstructure of emulsions stabilized by different emulsifiers: WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin), heated WPI and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) at 0 min and 120 min of gastric digestion.

3.3.3 Intestinal digestion

3.3.3.1 ζ-potential

After 120 min of simulated gastric digestion, chyme (partly digested food that passes from the stomach to the small intestine) was adjusted to pH 7 to stop pepsin activity and transferred to another flask for simulated intestinal digestion. They were mixed with simulated intestinal fluid (SIF) which contained various mineral salts, bile salts, and enzymes (including lipase and pancreatin) and their ζ-potentials were measured.

Generally, ζ-potentials went back to negative values (Figure 10) for all samples because protein had net negative charge at pH 7. WPI and HWPI were significantly less negatively charged than samples with pectin. The ζ-potentials of WPI and HWPI were also higher than their initial values which was expected since protein hydrolysis or displacement by bile salts at the interface could alter their surface charge property (Xu and others 2014b). Since adsorbed pectin are strongly negatively charged, the groups with pectin carried more negative charge than WPI and HWPI. There was no significant difference among samples with different pectin concentrations which could be from charge shielding effect induced by high ionic strength (140 mM) in SIF.

After 120 min of intestinal digestion, surface net charge of WPI and HWPI were even less negative. This may due to proteins being continually hydrolyzed while being displaced by bile salts and lipid digestion products (such as free fatty acids) which were less charged than the proteins (Torcello-Gómez and others 2011; Maldonado-Valderrama and others 2008; Mun and others 2007). On the other hand, although the net charge of emulsions containing pectin also decreased during intestinal digestion, these samples were more negatively charged than

emulsions without pectin after digestion. Previous research found that pectin was more difficult to be displaced from the interface by bile salts compared with protein (Verrijssen and others 2014b), thus pectin provided significant amount of charges to the oil-water interface during intestinal digestion. In summary, due to proteolysis, WPI and HWPI emulsions lost their net surface charge, while those containing pectin were still highly charged due to the effect of pectin.

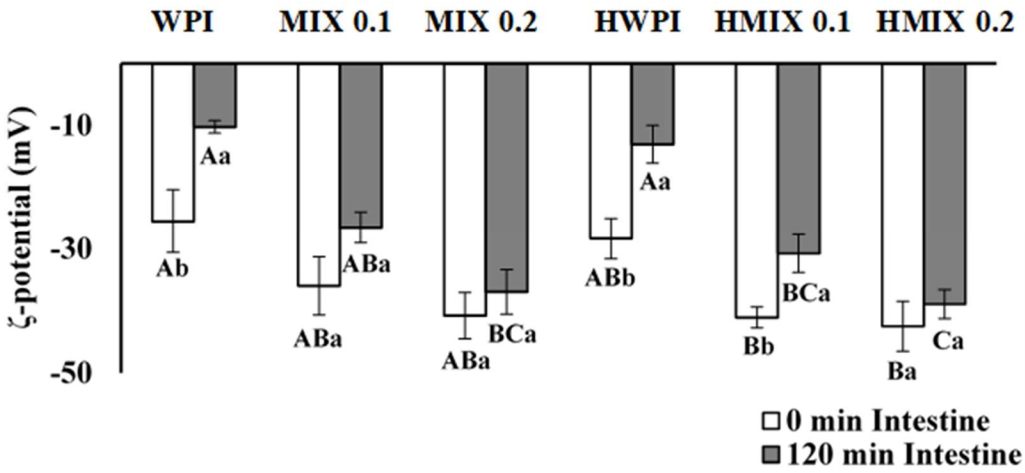

Figure 10 ζ-potential of emulsions stabilized by WPI, heated WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin) and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) at 0 min (White) and 120 min (Gray) of intestinal digestion.
Different capital letters indicate significant (P<0.05) difference between different samples at the same time point. Different lowercase letters indicate significant (P<0.05) difference between same sample at the different time points.

3.3.3.2 Emulsion structure

Structure of emulsions in intestinal digestion was also characterized by droplet size measurements (Figure 11 and 12) and confocal microscopy (Figure 13). After mixing with SIF,

WPI and HWPI emulsions underwent extensive coalescence as reflected by appreciably increase in droplet sizes and microscopic images. D_{43} increased from 8.2 μm to 11.3 μm and 2.6 μm to 8.4 μm for WPI and HWPI, respectively. As previously stated, this was mainly due to the shift of pH pass the pI which caused protein aggregation and proteolysis which caused the proteins to lose the ability to stabilize the emulsions (Malaki Nik and others 2011). Note that HWPI was also subjected to coalescence despite being quite resistant under gastric digestion.

On the other hand, mean droplet sizes of MIX and HMIX emulsions significantly decreased once they were mixed with SIF. Larger decrease in size was observed with MIX samples with D_{43} decreasing from 11.3 μm to 3.94 μm and 16.5 μm to 4.82 μm for MIX 0.1 and MIX 0.2, respectively. Microscopic images show flocculated oil droplets seen at the end of gastric digestion significantly dissociated from each other right after mixing with SIF. As pectin and protein both became negatively charged in SIF, the attraction between pectin and protein turned to repulsion (McClements 2015). Thus, flocculation was partly reversed driven by electrostatic repulsive force. However, coalescence is not reversible. As a result, D_{43} of HMIX emulsions were significantly smaller than MIX. During simulated intestinal digestion, proteins were gradually displaced by bile salts and being digested by pancreatin (Singh and Ye 2013). In emulsions containing pectin, bridging flocculated oil droplets dissociated to a greater extent (Figure 9), leading to a decrease in size. Because of surface charge and steric effect offered by adsorbed pectin, no further significant coalescence was observed (Verrijssen and others 2016). On the other hand, for WPI and HWPI emulsions, as interfacial structure was fully damaged, flocculated droplets coalesced to larger oil droplets (Figure 13) (Xu and others 2014b). Consistent results were shown in D_{43} (Figure 12).

After 120 min intestinal digestion, some very large oil droplets were observed in the digesta in WPI and HWPI, indicating that the oil was not completely digested. In contrast, for emulsions with pectin, undigested oil had much smaller sizes, especially HMIX. Microscopic images corresponded to the droplet size results. These samples remained undigested mainly due to the effect of pectin, which would be discussed in the next section.

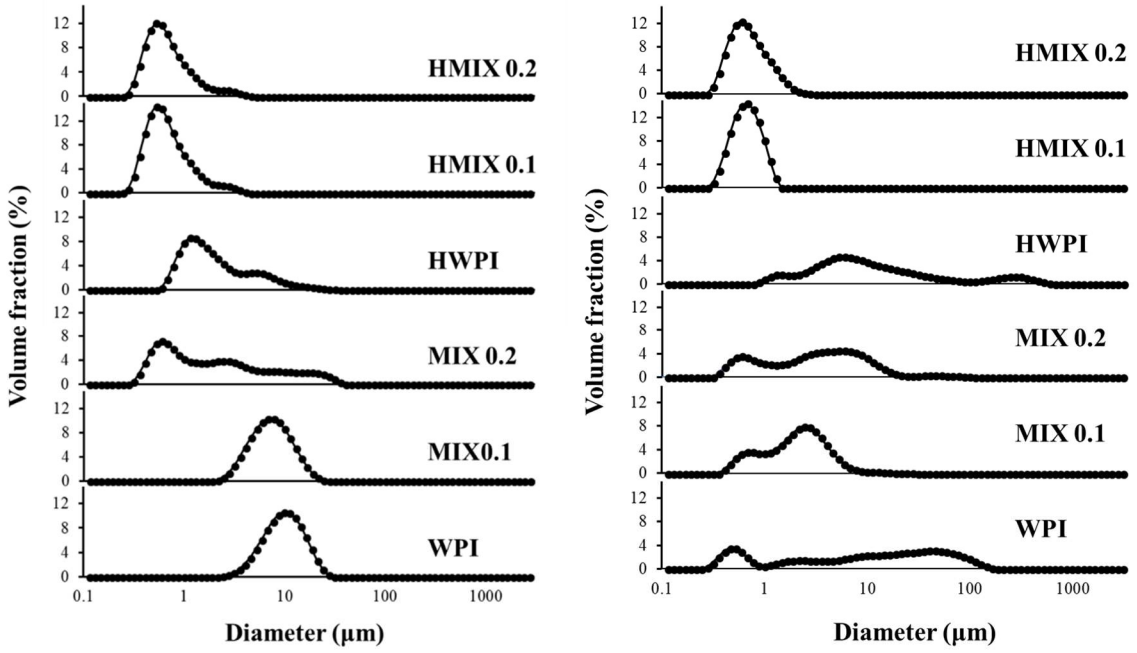

Figure 11 Particle size distribution of emulsions stabilized by WPI, heated WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin) and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) at the 0 min (A) and 120 min (B) of intestinal digestion.

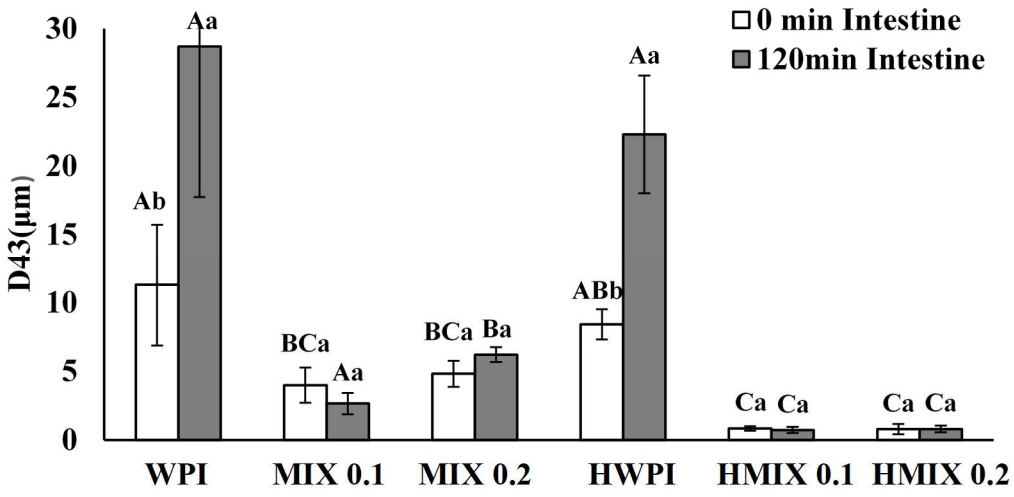

Figure 12 Mean particle size (D_{43}) of emulsions stabilized by WPI, heated WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin) and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) at the 0 min (White) and 120 min (Gray) of intestinal digestion.
Different capital letters indicate significant ($P<0.05$) difference between different samples at the same time point. Different lowercase letters indicate significant ($P<0.05$) difference between same sample at the different time points.

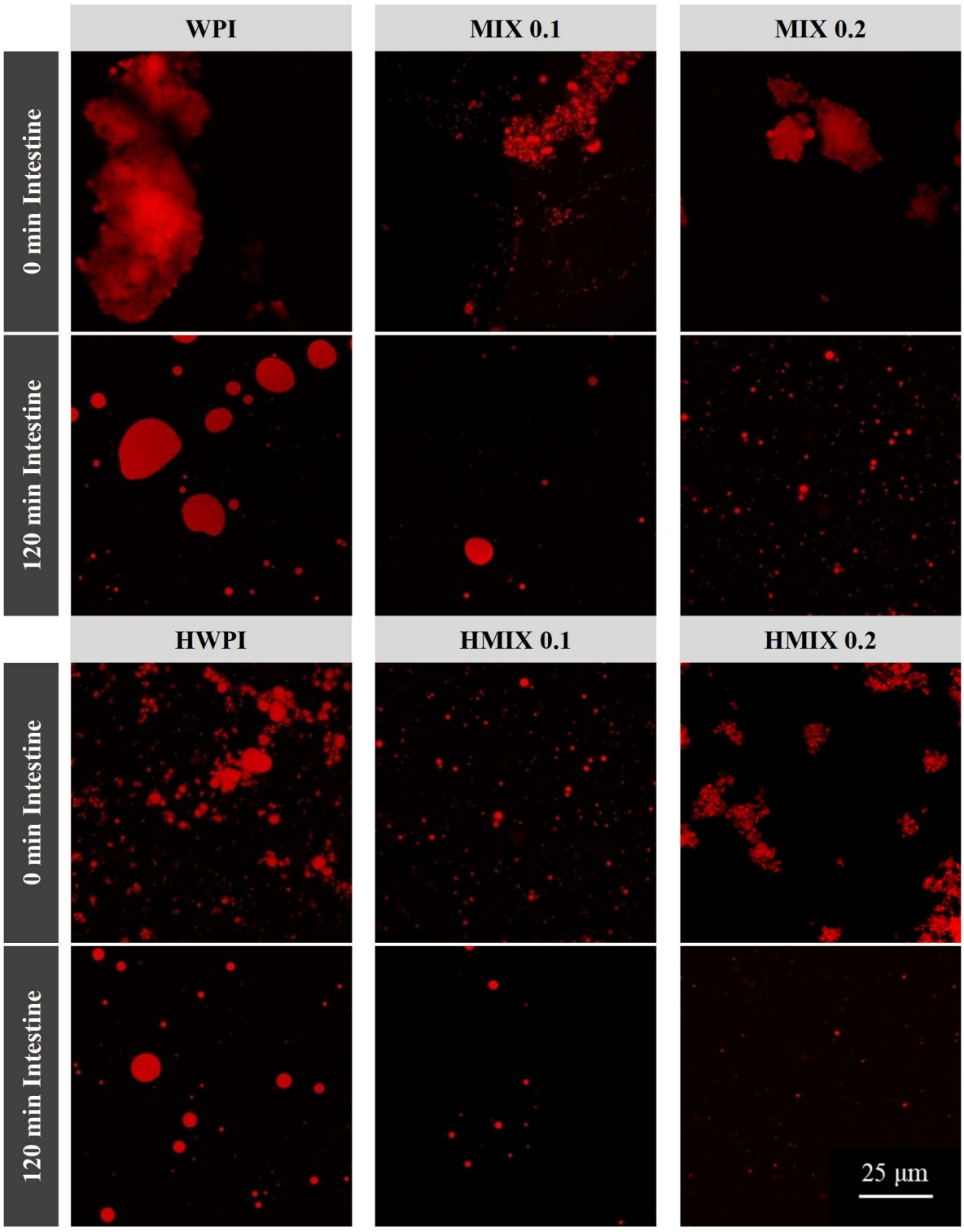

Figure 13 Microstructure of emulsions stabilized by different emulsifiers: WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin), heated WPI, and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) at 0 min and 120 min of intestinal digestion.

3.3.4 Digestion of lipid

Percent free fatty acid (FFA) release was measured for each sample at 12 time points, and the data was plotted (Figure 14). Then the data was fitted with a nonlinear regression. Two parameters were estimated (Table 1). Parameter C_f can be interpreted as the expected final FFA released (%) after 2 h of intestinal digestion. k can be interpreted as the reaction speed factor (Verkempinck and others 2018a) .

As shown in Table 1, increasing pectin led to lower C_f values and samples stabilized by unheated protein system also had lower C_f compared to heated system. Studies have reported that pectin can inhibit lipolysis in the intestine. The mechanism was fully illustrated in two aspects. First, pectin can bind to lipase, limiting their ability to hydrolyze lipid (Xu and others 2014b). Secondly, pectin decreases the displacement of proteins by bile salts, hence inhibiting the adsorption of lipase to the interface (Zhang and others 2015).

Droplet size (surface area) is also a key factor affecting lipid digestion (Zhang and others 2016a; Salvia-Trujillo and others 2013a; McClements and others 2008b). With smaller droplet size, oil droplet can provide larger reactive surface area to lipase and bile salts, then facilitate lipid digestion. As previously shown (Figure 14), MIX 0.1 and HMIX 0.1 had much smaller mean droplet sizes compared to HWPI and WPI. Thus, although they contained 0.1 wt.% of pectin, they had higher C_f. Similarly, HMIX emulsions had smaller droplet size and thus higher C_f compared to MIX emulsions at the same pectin concentration.

Pectin concentration also influenced the reaction rate as shown by lower k values with emulsions containing 0.2% pectin. HMIX 0.2 had the lowest k value among all samples. As stated above, pectin could hinder lipid digestion and thus decreased the rate the of FFA release. Similar finding was also reported by Verkempinck (2018a). However, it is interesting to note that

heated groups had slower FFA release rate than unheated groups. Yet mechanism behind that has not been fully unraveled.

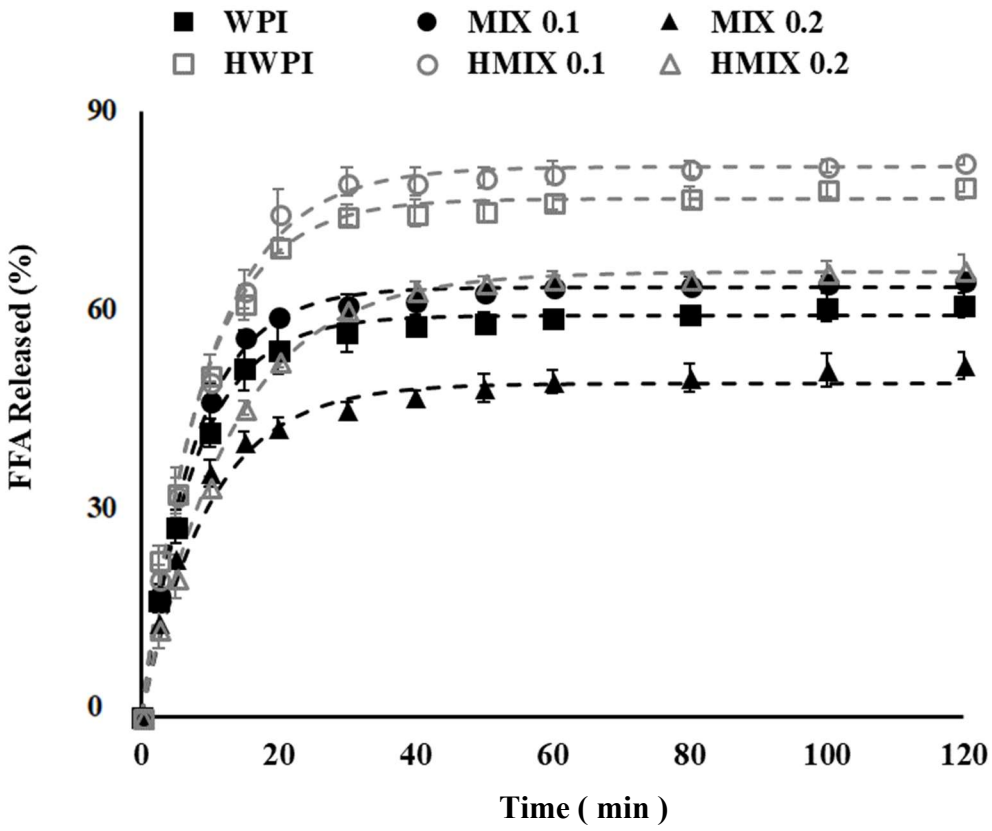

Figure 14 Free fatty acids released (%) from emulsions stabilized by different emulsifiers: WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin), heated WPI, and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) in intestinal digestion.

Table 1 Estimated kinetic parameters (±standard deviation) of a fractional conversion model for the emulsions stabilized by different emulsifiers WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin), heated WPI, and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin).

	C_f (%)	k (min^{-1})	Pseudo R^2
WPI	59.7±0.36^{d}	0.129±9.6E-3^{a}	0.98
MIX 0.1	64.0±1.81^{c}	0.130±7.6E-3^{a}	0.99
MIX 0.2	49.7±0.85^{e}	0.122±5.1E-3^{a}	0.99
HWPI	77.1±1.26^{b}	0.114±9.2E-3^{ab}	0.99
HMIX 0.1	81.1±0.13^{a}	0.098±5.9E-3^{bc}	0.99
HMIX 0.2	66.2±1.4^{c}	0.078±5.2E-3^{c}	0.97

3.3.5 β-carotene release

The release of β-carotene was measured at the end of *in-vitro* stomach and intestinal models. It should be noted that the extent of β-carotene release during the gastric and intestinal models have different meaning. During gastric digestion, flocculation and coalescence cause the breakdown of the emulsions. Potentially, β-carotene would be released from the emulsified oil droplets into the separated oil phase and floats to the top (Wang and others 2016). Apparently, that does not benefit the following digestion in the intestine. Studies have suggested that bulk oil (e.g., bulk oil phase that separated from the emulsified oil) has limited digestibility and bioactives that it carries will have lower bioaccessibility compared to emulsified oil (Zhang and others 2016a). In addition, some bioactives are sensitive to acidic environment, and pure β-carotene is reported to be rapidly degraded in the gastric phase during digestion (Donhowe and Kong 2014). So, releasing of emulsified lipid and β-carotene during gastric digestion is not favorable for the design of β-carotene delivery system. In contrast, the release of β-carotene

during intestinal digestion means that β-carotene is released from the digesta into the micelle phase. Percent β-carotene released into micelles is defined as bioaccessible β-carotene (Reboul and others 2006). Thus, it is desirable to prevent the release of β-carotene during gastric digestion and to increase the release during intestinal digestion.

As shown in Figure 15, 20% β-carotene was released in emulsions stabilized by WPI at the end of gastric digestion. Both heating and pectin influenced the release of β-carotene. Increasing pectin concentration led to lower release and emulsions stabilized by heated protein system also showed decreased release. Overall, β-carotene was most protected in HMIX 0.2 emulsion with only 8% release after gastric digestion. These results corresponded to previous results which showed destabilization of emulsion and severe coalescence in emulsions stabilized by unheated groups (WPI and MIX). It should be noted that we also observed phase separation and large bulk oil droplet floating on the top. For HMIX groups, strong interfacial layer led to emulsions that were more stable which resulted in the lowered release of β-carotene. Overall, results support that the release of β-carotene was related to the extent coalescence in the gastric phase as well as the oil droplet size. Flocculation without coalescence may contribute to stability of emulsion in gastric digestion in some extent.

Results after intestinal digestion revealed the highest amount of β-carotene release in HWPI and HMIX 0.1 emulsions. This can be attributed to different reasons. For HWPI, lipase bile salts can easily attach to oil-water interface to facilitate release of β-carotene. Further, compared with unheated WPI, HWPI was more stable during gastric digestion and the mean droplet size was relatively smaller, which could benefit the digestion of β-carotene. Maximum bioaccessible β-carotene observed in HMIX 0.1 can be mainly attributed to the smaller size (larger surface area) of oil droplets which provided more reactive area for lipase and bile salts.

With more lipid being digested, more β-carotene was solubilized in the micelles and became bioaccessible. However, higher pectin could inhibit lipid digestion leading to limited bioaccessibility. Therefore, although HMIX 0.2 and MIX 0.2 also had relatively good emulsion structure their β-carotene bioaccessibilities were lower.

Many studies have revealed that β-carotene bioaccessibility is highly related to digestion of lipid (Zhang and others 2016a; Zhang and others 2016b; Salvia-Trujillo and others 2013b). The mechanism is the micelle, where β-carotene need to be solubilized, is formed form lipid digestion product (e.g., free fatty acid) and bile salts. (Tyssandier and others 2001). To confirm the existence relation between lipid digestion and β-carotene bioaccessibility, a linear regression was determined between % β-carotene release and % FFA release. The R square was found to be 0.89, which suggested the good positive linear relation between β-carotene. This linear relation was also reported by other studies (Salvia-Trujillo and others 2013b; Qian and others 2012a). The regression equation suggests with 1% FFA released, 1.56 % of β-carotene would be released into micelle.

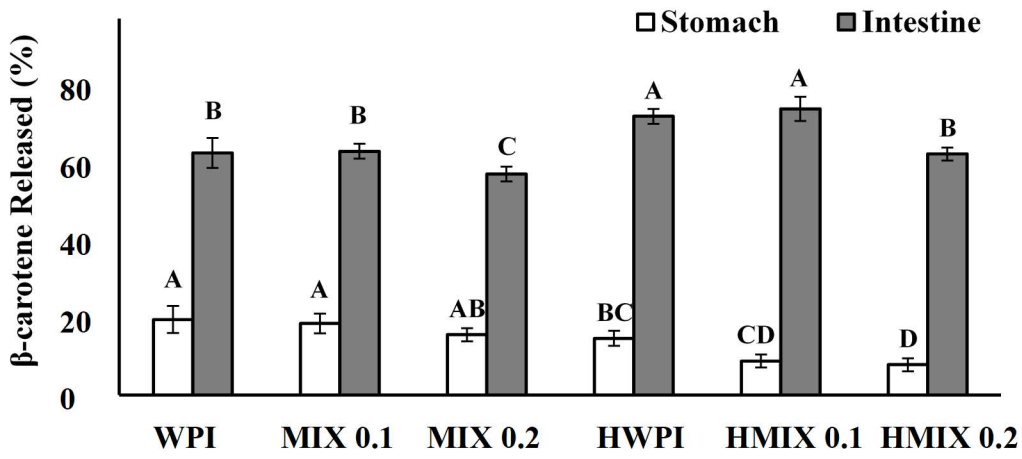

Figure 15 β-carotene released (%) of the emulsions stabilized by WPI, WPI-pectin mixture (with 0.1 wt.% or 0.2 wt.% pectin), heated WPI and WPI-pectin complex (with 0.1 wt.% or 0.2 wt.% pectin) in stimulated gastric (White) digestion and intestinal digestion (black). Different capital letters indicate significant (P<0.05) difference between different samples

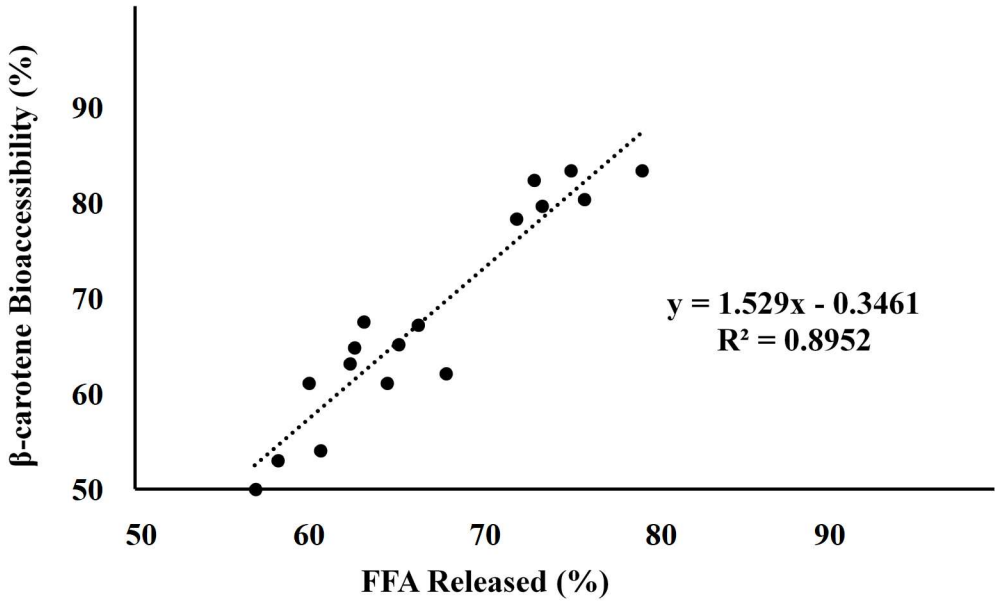

Figure 16 Correlation between the amount of free fatty acids released after the in vitro digestion and the bioaccessibility of β-carotene.

CHAPTER 4 CONCLUSIONS

This study has shown significant differences in digestion properties among emulsions stabilized by WPI, HWPI, MIX and HMIX. The differences were shown in aspects of emulsion structure, lipid digestibility and β-carotene bioaccessibility. Generally, the reasons can be attributed to the presence of pectin and heat-treatment. The ability of pectin to alter emulsion digestion could be due to i) electrostatic interaction with protein, ii) strong charge property and iii) steric hindrance. Heat treatment would cause formation of soluble aggregates or complexes between WPI and pectin which was shown to be beneficial in maintaining the integrity of the emulsion interfacial layer during digestion and leading to increased stability.

For lipid digestibility and β-carotene bioaccessibility, the results showed two trends. First, the more pectin presented, the less free fatty acid and β-carotene released. Second, increased stability of emulsion (e.g., maintaining small size and structural integrity) led to an increase in the amount of lipid and β-carotene digested. Finally, HWPI and HMIX 0.1 would be the best delivery systems.

Previously, most studies concluded that pectin would inhibit the digestion of lipid and was unfavorable for β-carotene bioaccessibility. This study suggests that by forming heated soluble complex and controlling pectin concentration, β-carotene bioaccessibility and lipid digestibility of emulsion containing pectin could be optimized. These results should provide more insight into the development of lipophilic bioactive delivery system.

APPENDIX

Emulsion	D_{43} (μm)	ζ-potential (mV)
WPI	0.767 ±0.029	-50.52 ±2.56
MIX 0.1	0.739 ±0.018	-50.23 ±2.79
MIX 0.2	0.826 ±0.095	-51.83 ±2.13
HWPI	0.751 ±0.027	-49.71 ±1.76
HMIX 0.1	0.768 ±0.021	-51.38 ±1.26
HMIX 0.2	0.763 ±0.045	-50.94 ±1.79

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