DEVELOPING CLEAN LABEL EMULSIFIER BASED ON WHEY PROTEIN AND

PECTIN COMPLEXES

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

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Developing clean label emulsifier based on whey protein and pectin complexes

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ABSTRACT

With increasing demand for clean label products, there is an urgent need to develop biopolymer-based emulsifiers and stabilizers. Proteins are excellent emulsifiers; however, their properties are limited at pH at or near isoelectric point (pI) and in the presence of salt. One approach to improve functional properties including emulsification properties of proteins is by interacting with polysaccharides. Protein-polysaccharide complex coacervates (e.g., pH < pI) have been extensively studied but much less focus has been given on complexation above pI. Previous studies have shown that heating protein and polysaccharides under a net repulsive interaction can induce the formation of soluble complex with improved functional properties such as acid-induced gelation and foaming properties. The overall objective of this research was to develop heated whey protein isolate and pectin complexes (H-complexes) formed at pH > pI with improved emulsification and stabilization properties.

In the first study, the effects of heating temperature (e.g., no heat, 75°C and 85°C) and pectin concentration on the physical and emulsification properties of WPI-pectin complexes were studied. Unheated WPI-pectin complexes (complexes) and heated WPI-pectin complexes (H-complexes) were formed by mixing or heating mixed biopolymers at pH 6.2.

Their particle sizes and zeta-potentials as well as emulsification properties and emulsion stability of o/w emulsions (5% oil) at pH 5.5 were evaluated. Results showed that formation of complexes or H-complexes led to altered protein aggregate sizes and increased negatively charged. Emulsification and stabilization properties were significantly improved as shown by decreased droplet sizes, decreased zeta potential and increased stability against creaming. Pectin concentration and heating conditions played important roles in the properties of the complexes. Emulsions stabilized by H-complexes formed at 85°C were more stable to creaming and heating.

In the second study, the goal was to optimize the emulsification properties of H-complexes in emulsions containing 20% oil by varying the heating pH and pectin concentration. H-complexes containing 3% protein and 0 to 0.6% pectin were formed at 85°C and pH 5.5, 5.8, or 6.2. Their emulsification properties were evaluated in o/w emulsions at pH 5.0. Results showed that, regardless of pH, increasing pectin concentration led to improved emulsification properties and stability. Emulsions stabilized by H-complexes formed at pH 5.5 and 5.8 showed better emulsification properties and stability compared to those at pH 6.2. Rheological properties of the emulsion were also influenced by complex formation pH.

It can be concluded that heated WPI-pectin complexes can be developed to have improved emulsification and emulsion stabilization properties. By varying pectin concentration, heating temperature and heating pH, the properties of the H-complexes can

be altered and their emulsification properties can be optimized such that they can be utilized in various applications.

CHAPTER 1 INTRODUCTION

1.1 Protein as natural emulsifier

Nowadays, natural food is a trend and also the option for consumer who is sensitive to some chemical food ingredients. Many consumers are now more motivated with health and well-being, especially healthy foods. Clean-label food is a new term for product that contains natural and simple ingredients without any artificial additives. Since natural ingredients can be much more profound to environment stresses than artificial ingredients due to their complex structure, thus producing all natural foods can be challenging.

There is increasing consumer consumption in clean label foods formulating with all-natural ingredients (McClements and Gumus, 2016, Ozturk and McClements, 2016). Therefore, replacement of synthetic ingredient with natural alternatives to meet consumer demands is needed. Several food products are considered as oil in water emulsions such as, beverages, sauces and dressings. Most emulsifiers used in the food industry are in synthetic form including, Tween 20, polysorbate 80 and mono-and di-glycerides of fatty acids. A great number of research have been studied on functional properties of natural emulsifiers in emulsion such as, plant protein (Can Karaca et al., 2015), animal proteins (Bouyer et al., 2012) and milk proteins (Livney, 2010). Caseinate and whey protein are major proteins in milk that have emulsifying properties due to their surface activity (Ozturk and McClements, 2016). To maintain emulsion stability, an emulsifier must rapidly adsorb and forms a strong membrane layer at the droplet surface. However, other ingredients and food process have a

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huge impact on proteins emulsifying properties which could lead to instability of emulsion, including pH, ionic strength, thermal process and freezing (McClements, 2004).

Whey protein can be used as alternative natural emulsifier because of its surface activity. Once whey protein is adsorbed on the oil droplets, if forms a layer that generates both electrostatic and to a lesser degree steric repulsion which help maintain emulsion stability (Ozturk and McClements, 2016). However, using protein to stabilize emulsion can be limited due to many environmental stresses. The effect of pH plays a big role on protein-stabilized emulsions properties. Protein surface are naturally charged because of amino acids. At protein isoelectric point (pI), protein molecules carry no net electrical charge and often precipitate. Protein surfaces charge depend on pH of the surrounding environment. At pH > pI, protein surfaces carry negative charge whereas at pH < pI protein surfaces are positive (Wagoner et al., 2016). Whey protein showed a potential to stabilize in emulsion at pH < pI and pH > pI but lead to flocculation at pI of whey protein (pH~5.2) due to lack of electrostatic repulsion (Demetriades et al., 1997, Chanamai and McClements, 2002, Huan et al., 2016d).

Pre-heating of protein before emulsification has a greatly impact on its functional properties. It has been reported that heated whey protein above denaturation temperature (> 60°C) lead to unfolding of protein structure. Denatured proteins (60-90°C, 1000s) reduced the ability of emulsifying due to large aggregates were formed (Millqvist-Fureby et al., 2001). On the contrary, the study from Dybowska (2011) showed that the emulsion stability was improved by pre-heated whey protein (80-95°C) compared to those stabilized by

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unheated protein. The contradiction on the effect of heating protein on emulsifying properties is depend on different conditions used in the studies such as, type and source of protein, concentration of protein, heating temperature and time and pH of the aqueous phase (Koupantsis and Kiosseoglou, 2009, Raikos, 2010)

1.2 Whey protein and polysaccharide interaction and complexation

Protein and polysaccharide interactions can be utilized to improve properties and stability in wide range food products. The formation of soluble whey protein and polysaccharide complexes is mainly driven by electrostatic interaction between oppositely charge from two different molecules (Jones and McClements, 2011). A number of research has focused on the formation of protein and polysaccharide soluble complexes at pH < pI where there is high electrostatic attraction between positively charge on protein surface and negatively charge from anionic polysaccharide (Jones et al., 2010b, Jones et al., 2010c, Salminen and Weiss, 2013). Nevertheless, the formation of protein and anionic polysaccharide soluble complexes at pH > pI where both two different molecules carry similar electrically charge still occurs. Although the protein surfaces are mostly negative charge but still there is positive charge localized on protein that can bind with anionic groups on polysaccharide (Dickinson, 2003, Vardhanabhuti et al., 2009b, Huan et al., 2016a).

Recently, studies have shown that formation of heated protein-polysaccharide soluble complexes enhances their stability against pH and ionic strength (Jones and McClements, 2008b, Jones et al., 2009a). During heating, unfolded protein molecules expose more charge groups which lead to further interaction with polysaccharide. Previous studies from our

group also reported improved functional properties of heated soluble whey protein isolate and polysaccharides formed at pH > pI (Zhang et al., 2012, Zhang et al., 2014a, Huan et al., 2016a, e).

1.3 Protein and polysaccharide interactions in emulsions

Interactions with polysaccharides will affect the protein functional properties, which including protein surface activity and emulsifying properties (Ye, 2008). Several studies on whey protein emulsifying properties with several types of polysaccharide such as, carboxymethylcellulose, pectin have shown improved emulsification properties and stability against creaming (Chanamai and McClements, 2002, Moreau et al., 2003, Guzey et al., 2004, Jourdain et al., 2008, Huan et al., 2016e).

The majority of research has focused on emulsification properties of protein and polysaccharide complexes formed at pH < pI, and only a small number of research investigated functionality of soluble complexes and especially heated soluble complexes formed at pH above pI. The major advantage of formation of heated soluble complexes at pH > pI is that they can be produced at higher protein concentration which allows the process to be practical in the industry. In this study, heated soluble complexes were formed by heating whey protein isolate (WPI) and pectin at pH > pI. Both WPI and pectin are the major ingredients used in foods and beverages and are considered clean-label. The objectives of this study were:

1. To investigate the effects of pectin concentration, pH and heating conditions on the physical properties of heated WPI-pectin soluble complexes.

2. To investigate the emulsification properties and stability of heated WPI-pectin soluble complexes in oil in water emulsions containing 5% or 20% oil at pH near pI.

3. To determine key factors that led to optimum emulsification properties of the heated WPI-pectin soluble complexes.

CHAPTER 2 LITERATURE REVIEW

2.1 Food Emulsions

Several commons foods such as salad dressings and mayonnaise are considered

emulsions which are two-phase systems of immiscible liquids. Food emulsions can exist as

either oil in water (o/w) where the oil is dispersed in the aqueous continuous phase or water

in oil (w/o) for which water is dispersed in the oil phase. Due to the large free energy at the

contact area between the oil and water phase, emulsion is thermodynamically unstable and

is susceptible to break down over time (McClements, 2011). The destabilization of

emulsion depends on variable physicochemical properties i.e. gravitational separation,

flocculation, coalescence, partial coalescence and Oswald ripening (McClements, 2007).

Droplet characteristics, such as concentration, size, charge, interfacial properties and

interactions directly affect the emulsion physicochemical properties (McClements, 2007).

The common unstable system found in o/w emulsion is gravitational separation or

creaming which is caused by differences in density between the oil droplets and the liquid

phase. Flocculation occurs when the droplet has enough attractive force with surrounding

droplets, leading to droplets association. Coalescence occurs when the droplets merge

together and form larger size, thus increase the rate of creaming rapidly while partial

coalescence is when droplet partly contacts with others. Oswald ripening is the process of

increasing mean size over time due to small oil molecules diffuse to larger molecules

(McClements, 2007).

Emulsifiers are surface active compounds that facilitate the formation and stability of emulsion over a time period (Ozturk and McClements, 2016). An effective emulsifier will adsorb on the droplet surface during homogenization process, then decreasing interfacial tension and form a strong repulsive interaction, such as steric and/or electrostatic repulsion. Importantly, the good emulsifier must be capable to stabilize emulsion in extensive processing such as, freezing, heating and stirring.

2.2 Whey protein

Bovine milk is a complex fluid with excellent nutrition profile to human diet. Milk proteins are composed of two major fractions: casein (80%) and whey protein (20%). Initially regarded as by-product from the cheese manufacturing, whey protein has become one of the major ingredients used in food, pharmaceutical and nutraceutical industries due to its nutritional and functional properties (Muro Urista et al., 2011). The major components of whey proteins are β-lactoglobulin, α-lactalbumin, immunoglobulin, serum albumin, lactoferrin and lysozyme (Livney, 2010). Processes such as membrane filtration, evaporation, and spray drying are used to transform whey proteins into value-added ingredients differing in protein concentration and composition. These include whey powder (~12-14% protein), whey protein concentrate (WPC, 35-80% protein), and whey protein isolate (WPI, ≥ 90% protein).

2.3 Roles of whey protein in emulsions

The preparation of food emulsions typically involves the use of emulsifier for facilitating the formation of emulsion and controlling product stability during storage. Synthetic small

molecular weight emulsifiers such as mono- and diglycerides are commonly used in many food products. However, with increasing consumer demand for clean label products, there is an urgent need to replace artificial emulsifiers with alternative natural emulsifiers. Whey protein has gained much interest because of its surface-active property which allows the protein molecules to adsorb at the interface between two immiscible liquid phases and decrease the surface tension (McClements, 2004, Ye, 2008, Livney, 2010). Adsorbed whey protein molecules undergo molecular structural changes and partially denature at the interface which leads to more exposure of sulfhydryl group and the formation of interfacial layer (Dickinson, 1998a). During homogenization, whey protein would rapidly form a thick layer at the interface which prevent droplets from coalescence or flocculation due to electrostatic and steric stabilization (Raikos, 2010).

Protein performances are based on their molecular and physicochemical characteristics such as protein sources, compositions, processing and storage conditions. Therefore, a slight difference in molecular properties could significantly change protein behavior. Protein emulsifying properties depend on their surface properties (e.g., adsorption rate, surface activity and surface loads) and electrical characteristics (McClements and Gumus, 2016). Whey protein is a globular protein and will change its conformation due to environmental change. In addition, globular proteins are less flexible than other types of protein and not stable through the thermal process that tend towards aggregation via hydrophobic attraction once they adsorb at the droplet surface (Kim et al., 2002).

External factors such as pH, ionic strength and heating are also important parameters affecting the emulsifying properties of whey protein. Protein has relatively poor stability at pH close to isoelectric points and tend to flocculation because of weak electrostatic repulsion (McClements, 2004). It has been reported that whey protein (0.5%) stabilized-emulsion at pH 5.2 (~ pI) showed significant increase in mean droplet size and had droplet net charge close to zero, suggesting droplet aggregation by attractive interactions between droplet (e.g. van der Waals and hydrophobic attraction) (Huan et al., 2016e).

Ionic strength plays an important role in determining emulsion properties through protein solubility and electrostatic properties. In the presence of $CaCl_2$ (> 20 mM), emulsion stabilized by whey protein (0.7%) was unstable to aggregation and promote flocculation at any pH value above isoelectric point (Chanamai and McClements, 2002). Adding Na^+ and Ca^{2+} in emulsion can alter droplet net charge due to the screening effect as well as these counter-ions can bind with droplet surface, thus reducing zeta-potentials (McClements, 2004).

Studies have reported that pre-heat treatment of protein beyond its denaturation temperature (> 65°C) can cause irreversible changes in whey protein structure which also greatly influences the emulsifying properties (Millqvist-Fureby et al., 2001). Globular whey protein can unfold and lose surface activity during heat treatment (Raikos, 2010). According to Millqvist-Fureby and others (2001), the emulsifying properties of heated whey protein (60-90°C, 1000 s) increased the destabilization due to large protein aggregates that inefficiently cover the droplets surface. On the other hand, Dybowksa (2011) reported that

pre-heating whey protein (80-90°C, 30 min) can form protein disulfide bond network at the droplet surface that helped stabilize emulsion. In agreement with the study from Ruffin and others (2014), heated whey protein (70-80°C, 5 and 30 min) increased emulsion stabilization as heating time increase with no any flocculation was observed. It can be concluded that the degree of protein denaturation can significantly influence protein emulsifying properties depending on heating temperature and time.

It is required that emulsifiers remain attach at the interface and continue providing stabilization functionality. When protein-stabilized emulsion is subjected to thermal process the protein will denature and interaction between adsorbed and non-adsorbed proteins in the aqueous phase can lead to increased droplet size and viscosity (Euston et al., 2000, Raikos, 2010). The study from Keowmaneechai and McClements (2002) reported that heating whey protein-stabilized emulsions at temperature ranging from 50-90°C resulted in increased viscosity and instability of emulsions. Further heat treatment (80-90°C) showed reducing in droplet aggregation due to complete denaturation of proteins leading to the formation of a more compact layer at the oil droplets (Raikos, 2010). Nevertheless, the mechanism behind the heating behavior of emulsion is still not clear, therefore there is a need of more research to investigate the emulsifying properties of protein in heated emulsion in various conditions.

2.4 Roles of polysaccharide in food emulsions

Most polysaccharides are extracted from plants and other natural sources, so they are considered consumer-friendly. Different types of polysaccharides have different molecular characteristics because of their compositions, molecular masses, degree of branching,

charge, hydrophobicity and polarity (McClements and Gumus, 2016). Some polysaccharides are used to stabilize emulsion by increasing the volume viscosity and thereby retarding the droplets motion. Some polysaccharides can act as emulsifier because of their surface activity such as, gum Arabic, guar gum and pectin (Dickinson, 2003). Once polysaccharide is adsorbed on droplet surface, they will form steric barrier that helped increase the stability of emulsion (Ozturk and McClements, 2016). The study from Chanamai and McClements (2002) reported that environmental changes such as, pH, ionic strength and temperature had little effect on emulsifying properties of polysaccharide (gum Arabic). Pectin was also reported to produce stable emulsions in the same way as gum Arabic with lower amount of usage due to some protein residues in pectin (Leroux et al., 2003).

Nonetheless, the disadvantage was it required high amount of polysaccharide to sufficiently cover the droplets due to their large molecular size (Ozturk and McClements, 2016). Moreover, the excess amount of polysaccharide present in emulsion could enhance the depletion flocculation which cause a number of adverse effects on creaming stability, appearance and enhance droplet coalescence (Chanamai and McClements, 2001). High concentration of polysaccharide is required to ensure a stable emulsion, as a result, those non-adsorbed polysaccharides will influence the osmotic pressure and increase the interaction between emulsion droplets that led to flocculation (Jenkins and Snowden, 1996).

2.5 Protein and polysaccharide interaction

Protein and polysaccharide are the common ingredients in many food systems. Their presence and interaction contribute to the structure, texture and stability of food. Due to their charge properties, protein and polysaccharide interactions and their effects on food properties and quality have been extensively studied. The mixing behavior of protein and polysaccharide is controlled by enthalpic effects. As the entropy energy exceeds the enthalpy energy, the two phases separation phenomena could occur (Doublier et al., 2000). Thermodynamic incompatibility or segregation phase separation exists when protein and polysaccharide have net repulsive interaction. Therefore, the system demixes into two phases, each being enriched with one type of the biopolymers. In contrast, when protein and polysaccharide show net attractive interaction, complexation occurs, resulting in the formation of soluble complexes or an associative phase separation. The two coexisting phases in associative phase separation have a solvent rich phase with very small amounts of biopolymers and a rich biopolymer phase forming coacervate (Syrbe, 1998). The interactions occur in solution either by complex coacervation or thermodynamic incompatibility are controlled by pH, ionic strength, conformation, charge density and protein and polysaccharide concentration (Ye, 2008).

In most cases, protein and polysaccharide interaction is primarily influenced by pH because of their functional side groups (i.e. amino and carboxylic groups) (Schmitt et al., 1998). Complex formation usually occurs at pH values < pI and at low ionic strength, where protein and anionic polysaccharide carry opposite net charges (Ye, 2008, Wagoner et al., 2016). However, at mildly acidic or neutral pH (pH > pI) where negatively charged

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functional groups mostly dominate, there are still some positive charges located on the surface that can interact with anionic polysaccharides (Dickinson, 2003).

Electrostatic complexation is usually a reversible process; however, studies have shown that the formation of protein and polysaccharide complexes by controlled thermal treatment can create new promising ingredients that are stability over the wide range of pH and ionic strength (Jones and McClements, 2008b, Jones et al., 2009a, Jones et al., 2010c). Heating globular proteins leads to conformation changes and molecular interactions which also affects the biopolymer structure (Jones et al., 2010b). Heated protein and polysaccharide complexes can be formed either by: 1) heating a solution of globular proteins above denaturation temperature and complexing with an ionic polysaccharide or 2) directly heating a solution of mixed biopolymers. Comparing the two approaches, complexes formed by mixing heated β-lactoglobulin with pectin via the first approach consist of a protein core surrounded by a pectin shell (Jones and McClements, 2008a). Complexes formed via the second approach would have smaller size and better pH stability (Jones and McClements, 2011). Heated solution of mixed biopolymers also showed higher polysaccharide surface coverage, thus increasing the biopolymer stability at low pH (pH < 4) and in addition of salt (200 mM NaCl) (Jones et al., 2010b).

The majority of research have focused on the formation of protein and polysaccharide soluble complexes at pH < pI where there is high electrostatic attraction between the biopolymers (Jones et al., 2010b, Jones et al., 2010c, Salminen and Weiss, 2013). Nevertheless, the formation of protein and anionic polysaccharide soluble complexes at pH

> pI where both two different molecules carry similar net charge still occurs. Although the protein surfaces are mostly negatively charged, there are positively charges localized on the protein surface that can bind with anionic groups on polysaccharide (Dickinson, 2003, Vardhanabhuti et al., 2009b, Huan et al., 2016a). Complexation in this pH range together with controlled heating could produce heated soluble complexes with improved functional properties. Recent studies reported that soluble complexes formed by heating whey protein and anionic polysaccharide at near neutral pH showed enhanced properties in acid-induced gels (Zhang et al., 2014a, Zhang and Vardhanabhuti, 2014, Huan et al., 2016b), foams (Zhengshan et al., 2015, O'Chiu and Vardhanabhuti, 2017), emulsions (Kim et al., 2006, Xu et al., 2012) and encapsulation (Chen and Subirade, 2006, Hong and McClements, 2007).

2.6 Protein and polysaccharide complexation in food emulsions

Proteins are known for the ability to stabilize emulsion and polysaccharides are known as a gelling and thickening agent. Since, there is growing interests in natural food consumption, one of the promising approach is using biopolymers such as proteins and polysaccharides as a building block to improve emulsifying properties (Matalanis et al., 2011).

Interaction between protein and polysaccharide can be utilized to enhance emulsion stability by inducing both steric and electrostatic stabilization as well as increasing continuous phase viscosity (Dickinson, 2009, Bouyer et al., 2012, Ruffin et al., 2014). There are two approaches in which emulsion droplets can be stabilized by protein and polysaccharide electrostatic complexes. The first method is to form a primary emulsion

using protein as emulsifier and then add a polysaccharide to adsorb on to the protein layer forming a "bilayer." The other method is called "mixed emulsion" which is achieved by forming an emulsion using mixed solution of protein and polysaccharide at pH where there is no interaction. Once the protein forms an adsorbed layer onto the oil droplets the pH is adjusted such that the polysaccharide will adsorb onto the protein layer (Dickinson, 2008). In comparing the two methods using sodium caseinate (CN) and dextran sulfate (DS), Jourdain and others (2008) showed that mixed emulsions had via the stability of mixed emulsion was greater than the emulsion prepared by bilayer technique. Similar result from (Huan et al., 2016e) reported stable acid-induced emulsion prepared by mixed system with whey protein isolate (WPI) and carboxymethylcellulose (CMC) soluble complexes (5 vol.% oil, 0.5 wt.% WPI, 0.08 wt.% CMC).

Controlling protein and polysaccharide ratio and polysaccharide concentration enhance the stability of emulsion. Stabilizing emulsion with non-adsorbed biopolymers can lead to depletion flocculation by osmotic pressure from narrow space between droplets that later promote the attractive interaction. In addition, the bridging flocculation can occur by electrostatic interaction between adsorbed protein and polysaccharide when polysaccharide is present in low concentration (McClements, 2004, Schmitt and Turgeon, 2011). Addition of very low pectin (< 0.02 wt.%) or (> 0.1 wt.%) in emulsion coated with β-lactoglobulin (1 wt.%) resulted in unstable emulsion due to depletion flocculation or the bridging flocculation (Cho and McClements, 2009).

In summary, it is important that the formation of complex has sufficient electrostatic and steric repulsion, thus the investigating of the optimum protein and polysaccharide ratio and polysaccharide concentration can be used to avoid bridging or depletion flocculation.

2.7 Heated protein and polysaccharide complexes and their emulsification properties

Protein and polysaccharide complexes can be sensitive to environmental conditions e.g., changing pH and increasing ionic strength. However, formation of protein and polysaccharide complexes through heat treatment shows better stability in a wide range of pH and ionic strength (Jones et al., 2010c).

Once an emulsion has been formed, it is important that it remains stable throughout food process and storage conditions. In the work from Salminen and Weiss (2014), heated whey protein and pectin complexes (85°C, 20 min) showed remarkable result in stabilization of emulsions at low pH (pH 3.0 - 4.5) with addition of salt up to 200 mM. The heated complexes also improved heat stability of emulsion with no aggregation was observed. Study from Xu and others (2012) reported that dry heated WPI and pectin (80°C, 5 h) increased the physical stability of β-carotene in emulsion and inhibited β-carotene degradation (5 vol.% oil, 0.5 wt.% WPI, 1 wt.% pectin). In addition, heated complex could form a thicker interfacial membrane compared to unheated complex, thus it can prevent coalescence during freeze-thaw stability.

2.8 Conclusion

It is important to develop biopolymer-based emulsifiers. Heated soluble complexes with higher degree of interactions between the biopolymers are expected to have better emulsification properties. To the best of our knowledge, no study has studied the emulsification properties of heated whey protein and polysaccharide complex formed at pH > pI where their interactions can be enhanced during heating. The overall goal of this thesis was to investigate the effect of complex formation temperature and pH as well as pectin concentration on emulsification properties of heated whey protein isolate and pectin complexes and to evaluate their potential as clean-label emulsifiers and stabilizers.

CHAPTER 3 MANUSCRIPT 1: FORMATION OF HEATED WPI-PECTIN COMPLEXES AT pH > pI WITH IMPROVED EMULSIFICATION PROPERTIES

3.1 Abstract

With increasing demand of clean-label products, there is an urgent need to develop biopolymer-based ingredients with improved emulsification and stabilization properties. Protein-polysaccharide complex coacervates (e.g., pH < pI) have been extensively studied but much less focus has been given on complexation above pI. The objective of this study was to develop heated whey protein isolate and pectin complexes (H-complexes) formed at pH > pI with improved emulsification and stabilization properties. H-complexes were formed by heating mixed 3 wt% WPI and pectin (0.1 or 0.3 wt%) at pH 6.2 and 75 or 85°C for 15 min. Emulsions were made at pH 7 before pH adjustment to 5.5. Final emulsions contained 5 wt% oil, 1.5 wt% protein and 0 to 0.15 wt% pectin. Emulsification properties were assessed by measuring droplet size, ζ-potential, rheological properties, creaming stability, and heat stability. Emulsification properties of H-complexes were also compared to unheated WPI (WPI) and heated WPI (H-WPI) as well as unheated complexes (complexes). Emulsions stabilized by WPI and H-WPI at 75°C and 85°C had the average droplets sizes of 393 ± 17.9, 434 ± 37.5 and 533 ± 34.7 nm, respectively with zeta potentials of -22.7 ± 1.2, -25.8 ± 0.9 and -28 ± 1.3, respectively. Complexation with pectin resulted in smaller droplet sizes, higher negative charge ($P < 0.05$). At 0.15% pectin in emulsion, the average droplet sizes ranged from 285 to 380 nm and zeta potentials ranged from -32.6 to -36.0 with no difference ($P > 0.05$) between complexes and H-complexes. Increased concentration of pectin in complexes and H-complexes exhibited more like Newtonian

fluid. Complexation with pectin also led to drastic increase in creaming stability with H-complexes showing the overall highest stability. At 0.05% pectin, complexes-stabilized emulsions showed separation on Day 23 while that stabilized by H-complexes were stable > 45 days. At 0.15% pectin, emulsions stabilized by both complexes and H-complexes were stable > 45 days. The major difference among complexes and H-complexes at 75°C and 85°C was in heat stability of the emulsions. While complexes and H-complexes at 75°C - stabilized emulsions formed gel after heating those stabilized by H-complexes at 85°C did not and their rheological properties remained the same. These results indicate that H-complexes have improved emulsification and stabilization properties and can be utilized in clean label applications.

3.2 Introduction

With increasing consumer demand for clean label products, there is a growing need for "clean" ingredients including emulsifier and stabilizer (Surh et al., 2006, McClements and Gumus, 2016, Ozturk and McClements, 2016). Protein is a natural emulsifier with excellent emulsifying properties at pH away from their isoelectric point (Dickinson, 2008). However, using protein alone may not sufficiently prolong emulsion stability during environmental stress such as pH, temperature, ionic strength and storage (McClements, 2004, Surh et al., 2006). Among different approaches that have been developed, the use of polysaccharides to improve the emulsification and emulsion stabilization properties of protein via electrostatic interactions has been extensively studied (Dickinson and Pawlowsky, 1997, Gu et al., 2004, Guzey and McClements, 2007, Jones et al., 2010b, Xu et al., 2012, Salminen and Weiss, 2014, Huan et al., 2016d). Protein and polysaccharide

complexes can stabilize emulsion droplets via two methods. In a 'bilayer emulsion' approach, a primary emulsion is prepared with the protein as an emulsifier, and the charged polysaccharide is added to produce a secondary emulsion with droplets having a protein-polysaccharide 'bilayer' surface coating. Alternatively, a 'mixed emulsion' is made by first preparing a solution of protein-polysaccharide complex at pH > pI before homogenizing with oil and acidifying to desirable pH (Jourdain et al., 2008, Huan et al., 2016d). At pH > pI, both biopolymers carry net negative charge which could typically lead to segregative phase separation (Tolstoguzov, 1986, Dickinson, 1998b, Turgeon et al., 2003); however, interactions still exist between the positively charged patches on the protein and negatively charged polysaccharides, resulting in the formation of soluble complex (Galazka et al., 1999, Vardhanabhuti et al., 2009a, Zhang et al., 2012). In mixed emulsion, the adsorption of protein-polysaccharide complex as well as additional interactions between the biopolymers at the interface could alter interfacial properties, leading to improved emulsification properties and stability (Jourdain et al., 2008, Jourdain et al., 2009, Huan et al., 2016d).

Heating solutions of biopolymer complexes above the denaturation temperature of the protein creates heated protein and polysaccharide complexes or particles (Gentes et al., 2010, Jones and McClements, 2011, Zhang et al., 2012). During heating, protein will unfold and expose some charged groups, which are available for further reactions. Physical and functional properties as well as stability of these complexes depend on the biopolymer ratio, polysaccharide type, pH, ionic strength, and heating conditions (Jones et al., 2009b, Gentes et al., 2010, Jones et al., 2010c, Jones and McClements, 2010, Zhang et al., 2012). (Jones and McClements, 2008a) reported that heating β-lactoglobulin and beet pectin

complexes at pH 5 at 83°C for 15 min could stabilize these complexes over pH 3 – 7. Gentes and others (2010) also demonstrated that severe heating conditions (85 and 90°C) allowed the stabilization of WPI and low methoxyl pectin complexes against pH change. The majority of research in this field has focused on complexation conditions at pH < pI where there are strong electrostatic attractions between the biopolymers. Heated whey protein isolate (WPI) and polysaccharides at pH > pI were previously studied by our group. The potential use of these heated complexes is justified because they show improved functional properties as compared to the individual macromolecules. Potential applications include acid-induced gel, foam and aerated gel (Zhang et al., 2014a, Zhang and Vardhanabhuti, 2014, Zhengshan et al., 2015, O'Chiu and Vardhanabhuti, 2017). Additional advantage of forming heated protein and polysaccharide complexes in this pH region is that they can be manufactured at higher protein concentration, allowing the technology to be applicable in the industry.

To the best of our knowledge, there has been no report that fully investigates the properties of heated protein and polysaccharide complexes in mixed emulsion. Heating condition influences the physical properties of the heated complexes; thus, it is likely to influence their emulsification properties as well. In this study, heated complexes were formed by heating WPI with low methoxyl pectin at pH > pI and their emulsification properties were investigated. The effect of pectin concentration and heating temperature were studied. Whey protein can undergo the molecular changes during the temperature above 70°C (Millqvist-Fureby et al., 2001, Dybowska, 2011); thus, heating temperatures at 75 and 85°C were selected and compared with unheated complexes. Finally, since most food

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emulsions become destabilize during thermal heating due to kinetic instability (Gu et al., 2005, Raikos, 2010), heat stability of emulsions was also determined.

3.3 Materials and Methods

3.3.1 Materials

Whey protein isolate was provided by Grande Custom Ingredients Group (Lomira, Wisconsin). According to the manufacturer, WPI contained 88.1% protein and 2.5% ash on a dry basis. Low methoxyl pectin (LM-12 CG) was provided by CP Kelco Inc. (Lille Skensved, Denmark). Phosphate buffer (5mM, pH 5.5 and 6.2) and all solutions were made with Milli-Q water (>18.2 MΩ/cm; Millipore, Billerica, MA). Commercial vegetable oil was purchased from local supermarket and all other reagents were of analytical grade.

3.3.2 Preparation of Stock Solutions

WPI stock solution (10% w/w) was prepared by slowly dissolving protein powder into water and kept stirred at room temperature for at least 2 h. Pectin stock solution (1% w/w) was prepared by slowly dissolving pectin powder into water at 60°C for 1 h under continuous stirring. After heating, the pectin stock solution was cooled to ambient temperature before weight readjustment if necessary. Both stock solutions were stored at 4°C overnight for complete hydration.

3.3.3 Preparation of Heated WPI-pectin complexes

WPI and pectin stock solutions were warmed up to room temperature (25 ± 1°C) for at least 1 h.

The two biopolymer solutions and water were mixed at an appropriate amount and the pH was adjusted to 6.2 with 0.1 M NaOH (50 μL at a time). Water was added such that the final solutions contained 3% protein and 0, 0.1, or 0.3% pectin. Samples (30 mL) were stirred for at least 2 h at room temperature before being heated at 75 or 85°C for 15 min. For WPI or complexes, solutions were prepared in a similar manner without heating.

3.3.4 Preparation of Emulsions

All oil-in-water emulsions containing 5% (w/w) oil, 1.5% (w/w) protein, and 0 to 0.15% (w/w) pectin were obtained by emulsification of oil with aqueous solution, e.g., WPI, heated WPI (H-WPI), unheated WPI-pectin complex (complexes) or heated WPI-pectin complex (H-complexes), through a 2-stage process. Appropriate amount of aqueous solution and water were mixed, and the pH of the mixture was adjusted to 7.0 (Guzey et al., 2004). After stirring for 1 h, appropriate amount of oil was added. Coarse emulsion was prepared by blending the mixture at 12,000 rpm for 15 s using a laboratory homogenizer, Ultra Turrax T-25 (IKA Instruments, Staufen, Germany). Final emulsion was obtained with an ultrasonic processor (Sonics VC 505, power 500 W, frequency 24 kHz; Sonics, Newtown, CT) with a sonotrode (3 mm, approximate length = 100 mm, titanium) at 40% amplitude for 3 min. Sodium azide (0.02%) was added as an antimicrobial agent. After emulsification, the emulsion was slowly acidified to pH 5.5 by adding 0.1 M HCl (50 μL at a time). The acidified emulsions were stirred for at least 1 h before analysis.

3.3.5 Particle size, Droplet Size and Zeta-Potential Measurement

Particle size and droplet size distributions as well as ζ-potential were determined using the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633-nm laser and 173°detection optics at 25°C. Aqueous solution was diluted to 0.3% w/w protein using 5 mM phosphate buffer at pH 6.2. Emulsion sample was diluted at a ratio of 1:1000 for size and 1:100 for zeta-potential using 5 mM phosphate buffer at pH 5.5 to prevent multiple scattering effects. An individual measurement was determined from the average of 5 readings taken on the same sample. All treatments were carried out in triplicate.

3.3.6 Rheological Properties Measurement

Rheological properties of fresh emulsions were measured using a Kinexus Pro Rheometer (Malvern Instruments Ltd., Worcestershire, UK) equipped with a cone (40-mm diameter, 4° angle) and plate geometry. Emulsion sample was loaded on a lower plate and the upper cone geometry was gently lowered to a gap of 0.05 mm. Flow behavior of the sample was conducted under a shear rate ramp from 0.1 s^{-1} to 200 s^{-1} at 25°C and under a solvent trap setting to prevent evaporation. Flow behavior index and consistency coefficient were calculated using the Power Law model. Each treatment was measured at least 3 times.

3.3.7 Creaming Stability of the Emulsions

Fresh emulsion sample (10 mL) was pipetted into a cylindrical glass tube (internal diameter = 16 mm, height = 100 mm). Subsequently, the tubes were sealed with Parafilm M film (Pechiney Plastic Packaging Company, Chicago, IL) to prevent evaporation. Emulsion

samples were stored quiescently at ambient temperature (~25°C) for 40 d. Emulsion

stability evolution was determined by measurement of height (millimeter units) of a

distinctive clear or semitransparent bottom serum phase layer on day 7, 14, 21 and 30 after

emulsion preparation. The extent of creaming was characterized by creaming index (CI, %)

= (HS/HT) x 100%, where HS is the height of the serum layer and HT is the initial height of

the emulsion. Each creaming index of an emulsion sample was recorded in triplicate.

3.3.8 Heat Stability of Emulsions

Freshly made emulsions were heated at 85°C for 15 minutes. Heated emulsions were

cooled to room temperature for 2 h before rheological analysis.

3.3.9 Statistical Analysis

Significance differences between treatments ($P < 0.05$) were determined by

ANOVA using Minitab (Version 17.1.0). Differences between means were determined

using Tukey's honestly significant difference (HSD) test.

3.4 Results and Discussion

3.4.1 Physical characteristics of H-complexes

We examined the effect of heating temperature (e.g., no heating, 75°C and 85°C)

and pectin concentration (e.g., 0, 0.15 and 0.3%) during heated complexes formation while

protein concentration (3%) and pH (6.2) were kept constant. WPI and complexes appeared

transparent without any precipitation (Figure 1a). Presumably, this was due to the

electrostatic repulsion among negatively charged protein molecules and/or the formation of

soluble complexes between protein and pectin (Jones et al., 2009b). Though both WPI and

pectin carry a net negative charge (pH > pI), the attraction between localized positively

charged patches on the protein can interact with anionic pectin (Dickinson, 1998b,

Vardhanabhuti et al., 2009a). It should be noted that the z-average diameters of the

unheated WPI and CPX samples were > 200 nm; however, the volume-weighted size

distribution showed the majority of the total peak (> 98%) was < 20 nm. This indicates the

presence of very small amounts of large particles, possibly protein aggregates, which

skewed the results. Thus, all unheated samples were filtered (0.2 μm) before measurements.

All heated samples were measured without filtration. Z-average diameters of complexes

were 10 ± 0.1 and 800 ± 88 nm (data not shown), respectively, which were in agreement

with the results from (Zhang et al., 2014a). Addition of pectin led to the formation of

soluble complexes having larger sizes as shown by the mean diameter of 15 ± 0.2 and 29 ±

1.6 nm at 0.1 and 0.3% pectin, respectively (data not shown). An increase in size suggested

a formation of complexes potentially with pectin layer on the protein surface.

WPI solutions turned translucent and opaque (Figures 1b and 1c) after heating at 75

and 85°C, respectively, due to denaturation and aggregation of WPI at >70°C (Jones et al.,

2009b). In the presence of pectin, H-complexes formed at 75°C showed decreasing turbidity

with increasing pectin concentration, while all heated samples at 85°C appeared opaque

without apparent difference. At 75°C, the z-average diameters of H-WPI decreased from

281 ± 18nm to 137 ± 21 and 187 ± 24 nm in the presence of 0.1 and 0.3% pectin,

respectively (Figure 2). It should be noted that H-complexes with 0.3% pectin at 75°C had

translucent solution and, similar to the unheated samples, the volume-weighted size

distribution showed the majority of the peak had an average diameter less than 50 nm.   This result is in agreement with the study from Donato and others (2009) reported heated β-lactoglobulin aggregates (70°C) at pH 5.9 were composed by small aggregates size between 50 to 70 nm.

At 85°C, the z-average diameters went from 557 ± 51 at 0% pectin to 311 ± 14 and 431 ± 9 nm in the presence of 0.1 and 0.3% pectin, respectively (Figure 2). These results indicate that interactions between WPI and pectin altered protein aggregation and H-complexes had smaller sizes compared to H-WPI. Similar results were reported by (Vardhanabhuti et al., 2009a). The changes in turbidity and particle sizes with temperature indicate changes in the structure of the aggregates or complexes. The slight increase in turbidity observed at 75°C was due to the unfolding and aggregation of a small fraction of the native protein molecules with increased thermal energy. Drastic increase in turbidity and particle sizes at 85°C was attributed to increased number of protein molecules engaging in heat aggregation and formation of larger aggregates. Complexation with pectin altered aggregation of protein as shown by decreased turbidity and mean diameters. Nonetheless, H-complexes at 85°C formed large particles that strongly affected the scattering light (Jones et al., 2010a). This implies that the structure and number of protein aggregates differed by the effect of heating between 75 and 85°C (Donato et al., 2009, Dybowska, 2011).

Zeta-potential (Figure 3) results showed that at pH 6.2, WPI and H-WPI at 75 and 85°C had the particle net charge of -19 ± 2.7, -21 ± 1.2 and -23 ± 1.9 mV, respectively. A slight increase in negative charge potential of WPI after heating has been reported (Ryan et

al., 2012, Salminen and Weiss, 2013). In all heated or unheated samples, addition of pectin led to a decrease in zeta-potential. At 0.3% pectin, complexes and H-complexes at 75 and 85°C had a zeta potential of -25 ± 3.5, -25 ± 2 and -28 ± 1.2, respectively. Change in zeta potential was likely due to binding of negatively charged pectin molecules to positively charged patches on the proteins before heating (e.g., complexes samples). During heating thermal energy could disturb the interactions but the presence of pectin molecules could alter protein aggregation as shown by the reduction in turbidity and particle sizes. At the same time, unfolding could expose hidden charged groups which could interact with pectin (Gentes et al., 2010). During cooling, pectin could then absorb to protein aggregates, resulting in aggregates being more negatively charged (e.g., H-complexes samples). It should be noted that changes in zeta potential between samples with 0.1 and 0.3% pectin were smaller, indicating limitation of additional binding at higher pectin concentration. It could be concluded from both particle size and zeta-potential results that, compared to WPI and complexes, the electrostatic interactions between protein and pectin were enhanced by forming H-complexes.
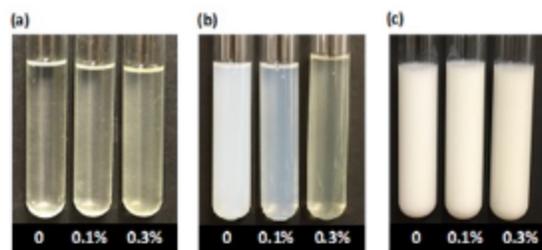
Figure 1 Effect of heating temperature and pectin concentration on the aggregation of WPI from left to right (0, 0.1, 0.3% pectin); unheated (a), heated at 75°C (b) and heated at 85°C (c).
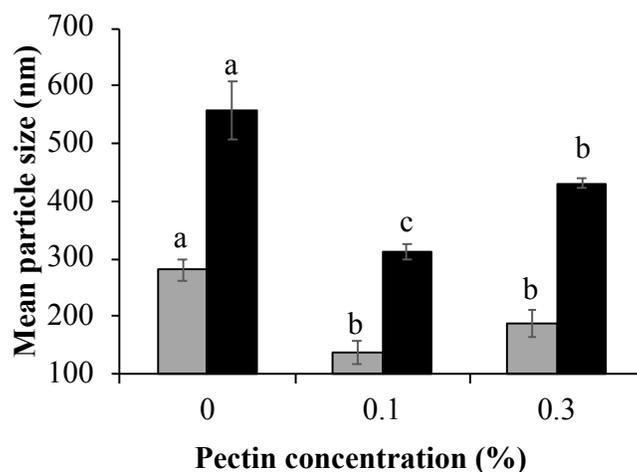
Figure 2 Mean particle size of WPI with different concentration of pectin and heating temperature; Heated at 75 ℃ (grey) and heated at 85 ℃ (black).

Results are the mean of three determinations. Different letters indicate significant differences (P < 0.05) between samples at the same temperature.
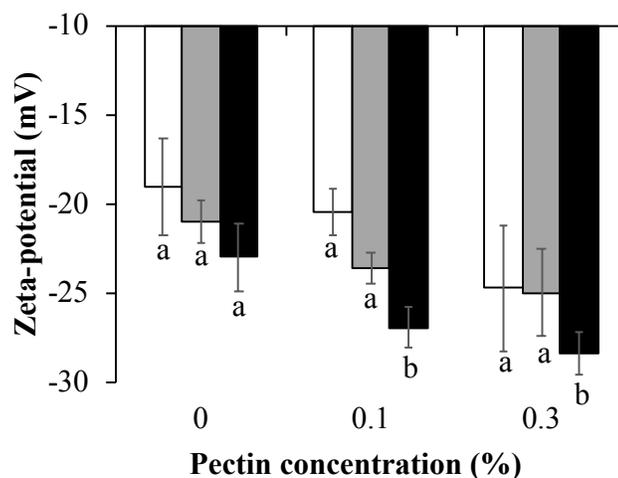
Figure 3 ζ- potential of WPI with different concentration of pectin and heating temperature; Unheated (white), heated at 75 ℃ (grey) and heated at 85 ℃ (black).

Results are the mean of three determinations. Different letters indicate significant differences (P < 0.05) between samples at the same temperature.

3.4.2 Emulsions

3.4.2.1 Zeta potential of emulsions

WPI, H-WPI, complexes or H-complexes were used as aqueous phase during

emulsion formation. The final emulsions contained 5%, 1.5% protein, and were at pH 5.5.

The final pectin concentrations in the emulsions were 0, 0.05 or 0.15% pectin which

corresponded to the protein solutions having 0, 0.1 and 0.3% pectin, respectively. In the

absence of pectin, the zeta potential of emulsions stabilized by WPI and H-WPI ranged

from -23 ± 1.3 to -28 ± 1.3 mV (Figure 4). The effect of heating showed that there was a

significant increase in droplet net charge when emulsions were stabilized by H-WPI ($P <$

0.05).

For mixed emulsions, the zeta potentials were significantly more negative ($P < 0.05$) and

were all below -30 mV. Higher negative charge was due to the interfacial adsorption of

complexes or H-complexes themselves being more negatively charged. In addition, it is

likely that the decreased charge of protein during pH adjustment to 5.5 allowed the

additional adsorption of free pectin in the aqueous phase that did not bind to the protein.

Similar results showing the charge properties of mixed emulsions have been reported

(Jourdain et al., 2008, Huan et al., 2016d). It should be noted that increasing pectin

concentration in complexes and H-complexes did not change the emulsion droplet charge,

suggesting that the droplet surface was saturated when emulsions contained 1.5% WPI and

0.05% pectin. At the same pectin concentration, there was no significant effect ($P > 0.05$) of

complex formation temperature (unheated, heated at 75 and 85°C) on the emulsion surface

charges. However, overall, the trend showed a slightly lower zeta potential in samples

stabilized by H-complexes formed at 85°C.
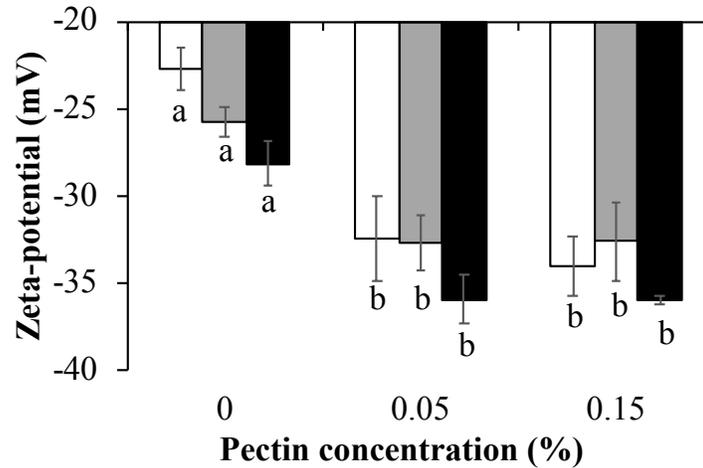
Figure 4 ζ- potential of emulsion droplets stabilized by WPI with different concentration of pectin and heating temperature; Unheated (white), heated at 75°C (grey) and heated at 85°C (black). Results are the mean of three determinations.

Different letters indicate significant differences (P < 0.05) between samples at the same temperature.

3.4.2.2 Mean droplet size of emulsions

The effect of pectin concentration and complex formation temperature on the mean droplet size of emulsions is presented in Figure 5. In the absence of pectin, mean droplet size of emulsions stabilized by WPI and H-WPI at 75 and 85°C were 393.4 ± 17.9, 434.3 ± 37.5 and 533 ± 34.7 nm, respectively. The effect of heating temperature showed significantly larger size ($P < 0.05$) when emulsion was stabilized by H-WPI at 85°C. It should be noted that these values were smaller than anticipated. In other studies, emulsions stabilized by unheated or heated WPI at pH near pI showed larger mean droplet diameters (Demetriades and McClements, 1998, Chanamai and McClements, 2002, Ruffin et al.,

2014, Huan et al., 2016d). This could be due to the different sources of protein used and the higher protein content in the emulsion. In addition, the emulsions were made at pH 6.2 before acidification; thus, the higher net charge at pH 6.2 could prevent droplet flocculation during emulsification, resulting in initial small droplet sizes. Droplet size measurement was conducted on freshly made emulsions; thus, flocculation after acidification was minimum.

The presence of pectin in mixed emulsions clearly reduced the mean droplet sizes and increased surface charge potential. Above 0.15% pectin in emulsion, those formed by complexes had smaller mean droplet sizes ($P < 0.05$) compared to WPI without pectin. Similarly, H-complexes stabilized emulsions also showed significantly decreased in mean droplet sizes ($P < 0.05$) compared to H-WPI. This is attributed to the adsorbed layer of higher negatively charged complexes on the droplet surface which provided sufficient electrostatic and steric repulsion that could prevent droplet aggregation. The effect of heating temperature on size showed that H-WPI and H-complexes at 85°C stabilized in emulsions had larger size ($P < 0.05$) compared to H-WPI and H-complexes at 75°C. Interestingly, we found that H-WPI and H-complexes at 75°C showed slightly higher net charge (more positive). As discussed in particle size of H-WPI and H-complexes at 75°C were fairly small and composed of aggregates and native proteins (Simmons et al., 2007). We presumably that because the presence of different types of particle (e.g., native protein, aggregates, complexes and H-complexes) were competed to form layer surrounded the oil droplet since the native WPI was more surface active and likely to adsorb on the droplet due to lowering the interfacial tension. The excess pectin could interact with dispersed protein in solution. This also implied that emulsion droplet stabilized by H-WPI and H-complexes at

75°C and 85°C had different properties because the overall structural and number of protein aggregated were differed by the effect of heating (Donato et al., 2009).
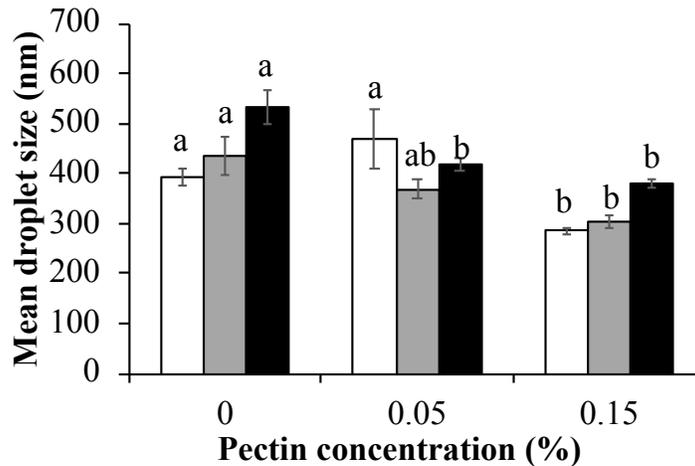
*Figure 5 Mean droplet size of emulsion droplets stabilized by WPI with different concentration of pectin and heating temperature; Unheated (white), heated at 75°C (grey), and heated at 85°C (black).*

Results are the mean of three determinations. Different letters indicate significant (P < 0.05) differences between samples at the same temperature.

3.4.2.3 Rheology of emulsions

As obtained from the Power Law model, consistency coefficient (K) and flow behavior index (n) are used to describe rheological properties of freshly made emulsions (Table 1). All emulsions exhibited low viscosity with a Newtonian or shear thinning behavior. The k and n values ranged from 0.002 to 0.009 Pa·s and 0.797 to 1.064, respectively. The apparent viscosity at 50 s^{-1} of all samples ranged from 0.001 to 0.005 mPa.s. WPI-stabilized emulsions exhibited a shear-thinning behavior. In general, n value of < 1 is determined as having shear thinning behavior which could be interpreted as the

disruption of flocs and decreased volume viscosity at increasing shear rate (Huan et al., 2016d). Emulsions formed with H-WPI at 75°C and at 85°C had increased n values to 0.857 and 0.905, respectively, while no change in the K was observed ($P > 0.05$).

Typically, addition of hydrocolloids could increase the viscosity of the continuous phase and resulted in decreased n and increased K values of the emulsions (Xu et al., 2012). In this study we found that the presence of pectin in complexes led to lower K and higher n values though the differences were not significant ($P > 0.05$). Emulsions formed by H-complexes at 75°C and 85°C also showed no significant difference in n and K compared to H-WPI at 75°C and 85°C, respectively.

Above 0.05% pectin, emulsions prepared with complexes and H-complexes increased K values which could be the effect of the excess of non-adsorbed pectin in aqueous phase. Interestingly, significantly higher K value was observed in those stabilized by H-complexes at 85°C with 0.15% pectin in emulsions.

The n values from emulsions stabilized by H-complexes at 75°C and 85°C are closed to 1, indicating that they behaved as Newtonian fluid. These results indicate that complexes and H-complexes led to the formation of small and non-flocculated droplets which was in agreement with the observed droplet sizes and particle charges.

Table 1 Power law model parameters for emulsions with different concentration of pectin and heating temperature

Heating temperature	Pectin (%)	K (Pa·s^n)	n	Viscosity at shear rate 50 s^{-1}
Unheated	0	0.007^a	0.797^a	0.004^a
	0.05	0.002^a	0.994^a	0.002^b
	0.15	0.004^a	1.010^a	0.004^{ab}
Heated at 75°C	0	0.009^a	0.857^a	0.004^b
	0.05	0.004^a	0.987^a	0.003^{ab}
	0.15	0.006^a	1.011^a	0.005^a
Heated at 85°C	0	0.002^{ab}	0.905^b	0.001^b
	0.05	0.002^b	1.105^a	0.002^b
	0.15	0.004^a	1.064^a	0.004^a

^{a,b}*Different letters within the same temperature during complex formation in emulsion indicate significant differences (P < 0.05).*
Consistency coefficient (k) and flow behavior index (n) were determined by fitting flow curves to the Power Law model. The data were the average from 3 measurements.

3.4.2.4 Creaming index

Figure 6 shows the effect of complex formation temperature and pectin concentration on creaming stability of emulsions during 30-day storage. Emulsions stabilized by WPI showed separation within 24 h (data not shown) despite the initial small droplet sizes. This could be due to weak electrostatic repulsion which is favorable for

droplet flocculation and coalescence (Gu et al., 2004). H-WPI- stabilized emulsions had

improved stability but creaming was observed on day 7 and 14 for H-WPI at 75°C and

85°C, respectively (Figures 6b and 6c). Improved emulsion stability of heated protein could

be due to the formation of thicker adsorbed layer consisting of protein aggregates

(Dybowska, 2011, Liang et al., 2017). Larger aggregates in H-WPI formed at 85°C could

provide better stability compared to H-WPI at 75°C.

In the presence of 0.05% pectin in emulsion, complexes and H-complexes at 75°C

formed emulsions with improved stability; however, creaming was observed on day 30 and

7, respectively. No creaming was observed in H-complexes at 85°C. Similar to the above,

due to their larger sizes, the H-complexes heated at 85°C could potentially form a stronger

adsorbed layer on the oil droplets. In addition, H-complexes at 85°C also showed lower

zeta potential though the difference was not significant. The approach to produce mixed

emulsion by complexation of unheated protein and polysaccharide has already been shown

to improve emulsions stability. Thus, improved creaming stability was expected. The

significant finding in this study is that, at lower pectin concentration, using H-complexes

formed at 85°C resulted in the most stable emulsions.
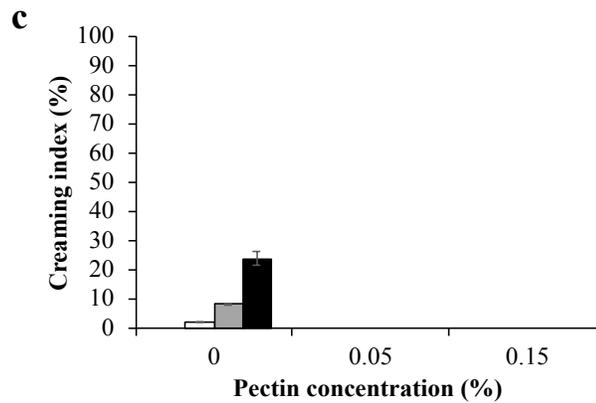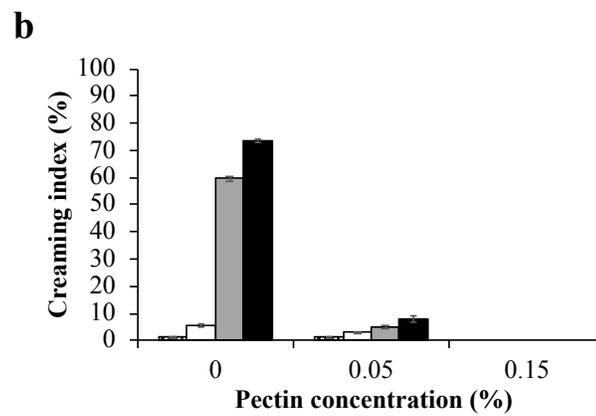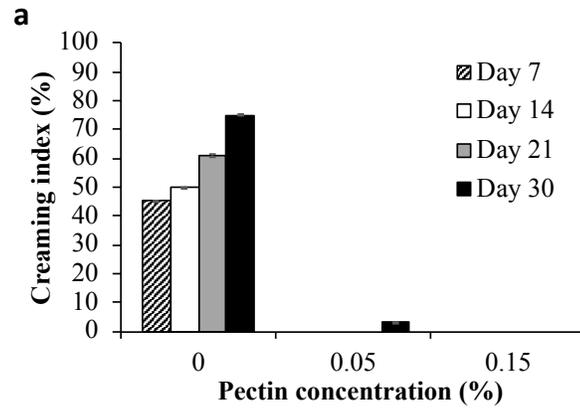
Figure 6 Creaming stability of emulsions prepared with different concentration of pectin and heating temperature during complex formation stored for 30 d at room temperature; Unheated (a), heated at 75℃ (b) and heated at 85℃ (c)

3.4.2.5 Heat stability of emulsions

Due to the lack of stability against creaming, emulsions stabilized by WPI or H-WPI were not tested for their heat stability. Regardless of pectin concentration, emulsions stabilized by complexes as well as H-complexes formed at 75°C all turned into weak gel after heating. In emulsions containing complexes, unfolding of native protein during heating expose the hydrophobic surface which promotes the interaction and aggregation between droplet and non-adsorbed protein (Euston et al., 2000, McClements, 2004, Çakır-Fuller, 2015). Extensive protein-protein interactions led to flocculation and eventually gelation. For H-complexes at 75°C, there could be some undenatured protein present while complete denaturation occurred in H-complexes at 85°C. Thus, unfolding as well as protein-protein interactions and gelation could occur in emulsions stabilized by H-complexes at 75°C. These samples were not further characterized.

Emulsions stabilized by H-complexes formed at 85°C maintained their fluid properties after heating. Size, zeta potential and rheological properties of these samples were characterized and compared to their properties before heating (Table 2). All emulsions stabilized by H-complexes at 85°C showed no significant change ($P > 0.05$) in zeta-potential and rheological properties (e.g., n and K) after the emulsions were heated while there was a slight increase in mean droplet sizes ($P < 0.05$). It has been suggested that large mean droplet sizes are protein aggregates from heating (Euston et al., 2000). However, the increase in droplet size was not enough to cause any significant changes in the rheological properties of emulsion. As shown by n and K values, emulsions were stable against flocculation and maintained Newtonian behavior with low viscosity. The apparent

viscosity at 50^{-1} of all samples showed low viscosity and ranged from 0.002 to 0.004 mPa.s.

Creaming stability experiment indicated that all emulsions were stable for more than 30 days (data not shown). These results suggested that H-complexes at 85°C could possibly form a relatively strong interaction between whey protein and pectin that improved emulsifying activity and provided steric force (Gu et al., 2005, Setiowati et al., 2017).

Table 2 Zeta-potential, mean droplet size (Z-average) and Power law model parameters of H-complexes formed at 85° C

stabilized in emulsions and heated emulsions (85° C, 15 min)

H-complexes at 85°C	Pectin (%)	Zeta-potential	Z-average	K (Pa·s^n)	n	Viscosity at shear rate 50 s^{-1}
Emulsion	0.05	-35.9 ± 1.4^a	419.3 ± 12.0^b	0.002^a	1.105^a	0.002^a
	0.15	-36.0 ± 0.3^a	380.4 ± 7.6^b	0.004^a	1.064^a	0.004^a
Heated emulsion	0.05	-34.3 ± 1.2^a	562.6 ± 4.7^a	0.003^a	0.942^a	0.002^a
	0.15	-35.3 ± 1.0^a	516.4 ± 6.7^a	0.004^a	1.019^a	0.004^a

[a,b]*Different letters within the same pectin concentration in emulsion indicate significant differences (P < 0.05).*

Consistency coefficient (k) and flow behavior index (n) were determined by fitting flow curves to the Power Law model.

3.4.3 Conclusion

Formation of complexes or H-complexes led to an increase in negative charge potential and, in case of H-complexes, altering the formation of protein aggregates. Pectin concentration was the major factor affecting the charge of the complexes while heating temperature mainly influenced the sizes of the H-complexes. Using complexes or H-complexes to stabilize the O/W emulsions in mixed emulsion approach resulted in emulsions having smaller droplet sizes, increased negative charge and improved stability against creaming. Emulsion properties and stability depended on pectin concentration as well as heating temperature. Only emulsions stabilized by H-complexes formed at 85°C were stable after heating. In conclusion, H-complexes formed at 85°C with sufficient amount of pectin are promising ingredients to be utilized in emulsions at near pI.

CHAPTER 4 MANUSCRIPT 2: OPTIMIZING THE EMULSIFICATION PROPERTIES OF HEATED WPI-PECTIN COMPLEXES FOR EMULSION CONTAINING 20% OIL AT pH 5.0

4.1 Abstract

There has been increasing interest in developing food ingredients for clean label applications. We have previously shown that heated whey protein and pectin complexes (H-complexes) formed at pH above pI have improved emulsification properties and stability when emulsions contained 5% oil. However, it is not fully understood whether these H-complexes could stabilize emulsions containing higher oil content as in sauces and salad dressings. The objective of this study was to optimize the emulsification properties of H-complexes in emulsions containing 20% oil at pH 5.0. H-complexes were formed by heating mixed 3 wt% WPI and pectin (0, 0.3, 0.45 wt%) at pH 5.5, 5.8 and 6.2 at 85°C for 15 min. Emulsions were made, followed by pH adjustment to 5.0. Final emulsions contained 20 wt% oil, 2 wt% protein and 0 to 0.3 wt% pectin. Emulsification properties were assessed by measuring droplet size, ζ-potential, rheological properties and creaming stability. Emulsions stabilized by heated WPI without pectin (H-WPI) had the average droplets sizes > 36 μm and zeta potentials ranging from -27.2 to -19.8 mV. They were not stable and separated into two layers within a few hours. H-complexes-stabilized emulsions showed significant improvement in emulsification properties and stability. Mean droplet sizes significantly decreased ($p < 0.05$) and ranged from 1.6 to 21 μm while droplets became more negatively charged with zeta potential ranging from -37 to -40.9 mV. Both heating pH and pectin concentration during H-complexes formation played important roles on the

emulsification properties of the H-complexes. The most stable emulsions (> 30 days) were those stabilized by H-complexes formed with 0.45% pectin at heating pH of 5.5 and 5.8. Formation pH also influenced the rheological behavior of the emulsions with those stabilized by H-complexes formed at pH 6.2 being more viscous. These results indicate that emulsification properties of heated WPI and pectin complexes formed at pH > pI can be optimized to stabilize emulsions containing higher oil content. They can be utilized as clean-label ingredients in applications such as sauces and dressings.

4.2 Introduction

Whey protein is one of the major emulsifiers used in foods due to its surface-active properties and its emulsifying properties have been extensively studied (Phillips et al., 1994, Dickinson, 1997) (Demetriades et al., 1997, Dickinson, 1999, McClements, 2004). Factors that influence the ability of whey protein to form stable emulsions include emulsion composition and environmental conditions (Dickinson, 1992). In products where the conditions promote instability, protein modification or addition of other ingredients such as polysaccharides are needed to improve protein properties and to help stabilize the emulsion via other mechanism (Chanamai and McClements, 2002). Protein and polysaccharide interactions play an important role in the structure and stability of food products including food emulsions. While they provide textural and stability advantages their presence does not require special legislation and is label-friendly.

Typically, protein and polysaccharide interactions are electrostatically driven. At pH near pI, where both polymers carry opposite charge, complexation between the biopolymers

can be utilized to improve emulsification properties of the proteins especially under unfavorable conditions for protein functionalities (e.g., pH at or near pI or increased ionic strength) (Dickinson, 2009, Schmitt and Turgeon, 2011). Nonetheless, even when the net interaction between protein and polysaccharide is repulsive (e.g., pH > pI) soluble complex formation can still occur (Zhang et al., 2014a, Wagoner and Foegeding, 2017). When heated together at pH > pI globular protein molecules unfold and expose more positively charged groups that can interact with anionic polysaccharide forming heated soluble complexes (Vardhanabhuti et al., 2009b, Zhang et al., 2012). Formation of heated complexes results in increased negatively charge potential and altered aggregates structure. Previous work in our laboratory show that heated soluble complexes between whey protein and polysaccharides formed at pH > pI have improved functional properties including heat stability, acid-induced gelation, aerated gel, intragastric gelation and foaming properties (Vardhanabhuti et al., 2009b, Zhang et al., 2014a, Zhang et al., 2014b, Zhengshan et al., 2015, Huan et al., 2016a, O'Chiu and Vardhanabhuti, 2017)

In emulsions, according to Dickinson (1988), the best way to adsorb polysaccharides onto interfaces is to link them to proteins. A polysaccharide that is thermodynamically incompatible with adsorbed protein can be distributed at the interface only if it can interact with the protein. Complexation between proteins and polysaccharides at the interface can improve steric stabilization as long as there is no bridging flocculation. In our recent study, we found that heated whey protein isolate and pectin soluble complexes formed at near neutral pH can be used to improve emulsification properties, as well as stability against creaming and heating of emulsions containing 5% oil at pH 5.5. Our previous work also

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showed that heating pH during complex formation influenced the physical properties of the complex but it was not clear how those would impact their emulsification properties. In additional, it was not known whether improved emulsification properties would be observed when emulsions contained higher oil content. The aim of this study was to investigate the effect of pH during the formation of heated whey protein isolate and pectin complexes on their emulsification properties in 20% oil emulsions at pH 5.0.

4.3 Materials and Methods

4.3.1 Materials

Whey protein isolate (Grande Ultra 9100) was provided by Grande Custom Ingredients Group (Lomira, WI). According to the manufacturer, WPI contained 88.1% protein and 2.5% ash on a dry basis. Pectin (GENU® LM-12 CG) was provided by CP Kelco Inc. (Lille Skensved, Denmark). Citrate-Phosphate buffer (5mM, pH 5.0, 5.5, 5.8 and 6.2) and all solutions were prepared with Milli-Q water (>18.2 MΩ/cm; Millipore, Billerica, MA). Commercial vegetable oil was purchased from local supermarket and all other reagents were of analytical grade.

4.3.2 Preparation of Stock Solutions

WPI stock solution (10% w/w) was prepared by slowly dissolving protein powder into water and kept stirred at room temperature for at least 2 h. Pectin stock solution (1% w/w) was prepared by slowly dissolving pectin powder into water at 60°C for 1 h under continuous stirring. After heating, the pectin stock solution was cooled to ambient temperature before weight readjustment if necessary. Both stock solutions were stored at

4°C overnight for complete hydration.

4.3.3 Preparation of Heated WPI-pectin complexes

WPI and pectin stock solutions were warmed up to room temperature (25 ± 1°C) for at least 1 h. The two biopolymer solutions and water were mixed at an appropriate amount and the pH was adjusted to 5.5, 5.8 and 6.2 with 0.1 M HCl or NaOH (50 μL at a time). Water was added such that the final solutions contained 3% protein and 0, 0.15, 0.3, 0.45 and 0.6% pectin. Samples (30 mL) were stirred for at least 2 h at room temperature before being heated at 85°C for 15 min.

4.3.4 Preparation of Emulsions

All oil-in-water emulsions containing 20% (w/w) oil, 2% (w/w) protein, and 0 to 0.3% (w/w) pectin were obtained by emulsification of oil with aqueous heated WPI (H-WPI) or heated WPI-pectin complex (H-complexes) solutions through a 2-stage process. Appropriate amount of aqueous solution and water were mixed. After stirring for 1 h, appropriate amount of oil was added. Coarse emulsion was prepared by blending the mixture at 12,000 rpm for 60 s using a laboratory homogenizer, Ultra Turrax T-25 (IKA Instruments, Staufen, Germany). Final emulsion was obtained by using an ultrasonic processor (Sonics VC 505, power 500 W, frequency 24 kHz; Sonics, Newtown, CT) with a sonotrode (3 mm, approximate length = 100 mm, titanium) at 40% amplitude for 3 min. Sodium azide (0.02%) was added as an antimicrobial agent. After emulsification, the emulsion was slowly acidified to pH 5.0 by adding 0.1 M HCl (50 μL at a time). The acidified emulsions were stirred for at least 1 h before analysis.

4.3.5 Particle Size and Zeta-Potential Measurement of Heated WPI-pectin complexes

Particle size and ζ-potential were determined with the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633-nm laser and 173°detection optics at 25°C. Each sample was diluted at a ratio of 1:100 for size and 1:10 for zeta-potential using 5 mM citrate-phosphate buffer at pH 5.5, 5.8 and 6.2 to prevent multiple scattering effects. An individual ζ-potential measurement was determined from the average of 5 readings taken on the same sample. All the measurements were carried out in triplicates.

4.3.6 Droplet Size and Zeta-Potential Measurement of emulsion

Droplet size distribution was determined with the Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK) and ζ-potential were determined with the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Samples were measured using the refractive indexes of 1.456 and 1.33 for oil and water, respectively. Each sample was diluted using 5 mM citrate-phosphate buffer at pH 5.0 to prevent multiple scattering effects. The droplet size was reported as the mean particle diameter d_{43}. All the measurements were carried out at least 3 replicates.

4.3.7 Rheological Properties Measurement

Rheological properties of fresh emulsions were measured using a Kinexus Pro Rheometer (Malvern Instruments Ltd.) equipped with a cone (40-mm diameter, 4° angle) and plate geometry. Emulsion sample was loaded on a lower plate and the upper cone

geometry was gently lowered to a gap of 0.05 mm. Flow behavior of the sample was conducted under a shear rate ramp from 0.1/s to 200/s at 25°C and under a solvent trap setting to prevent evaporation. Flow behavior index and consistency coefficient were calculated using the Power law model. Each treatment was measured at least 3 times.

4.3.8 Creaming Stability

Fresh emulsion sample (10 mL) was pipetted into a cylindrical glass tube (internal diameter = 16 mm, height = 100 mm). Subsequently, the tubes were sealed with Parafilm M film (Pechiney Plastic Packaging Company, Chicago, IL) to prevent evaporation. Emulsion samples were stored quiescently at ambient temperature (~25°C) for 40 d. Emulsion stability evolution was determined by measurement of height (millimeter units) of a distinctive clear or semitransparent bottom serum phase layer on day 7, 14, 21 and 30 after emulsion preparation. The extent of creaming was characterized by creaming index (CI, %) = (HS/HT) Å~ 100%, where HS is the height of the serum layer and HT is the initial height of the emulsion. Each creaming index of an emulsion sample was recorded in 3 replicates.

4.3.9 Statistical Analysis

Significance differences between treatments ($P < 0.05$) were determined by ANOVA using Minitab (Version 17.1.0). Differences between means were determined using Tukey's honestly significant difference (HSD) test.

4.4 Results and Discussion

4.4.1 Characterization of heated WPI-pectin complexes

In order to identify the optimum condition (e.g., pH and pectin concentration) during H-complexes formation that could stabilize o/w emulsion containing 20% oil at pH 5, the impact of solution pH and pectin concentration on WPI-pectin complexation was investigated. After heating, H-WPI and H-complexes at pH 5.5, 5.8 and 6.2 were all turned opaque due to protein-protein aggregation when heated above denaturation temperature (Salminen and Weiss, 2013). H-complexes remained turbid after samples were cooled, indicating that the aggregation was irreversible.

As shown in Figure 7, the zeta potentials of H-WPI were -15.2 ± 2.9, -16.6 ± 2.1 and -22.9 ± 1.9 mV at pH 5.5, 5.8, and 6.2, respectively. Increasing pectin concentration resulted in significantly lower zeta potential ($P < 0.05$) in all samples, indicating that pectin molecules were able to bind to localized cationic area on the protein via electrostatic attraction even when they both carried net negative charge (e.g., pH > pI). The largest degree of interaction was at pH 5.5 as observed by the largest decrease in zeta potential. Though the proteins themselves were less negatively charged at this pH, the decrease in charge repulsion allowed more interactions between WPI and pectin, resulting in the most negatively charged complexes. Smaller change in zeta potential of samples at pH 6.2 suggested limited interaction due to higher charge repulsion between the two biopolymers.

In the absence of pectin, z-average diameters of H-WPI were 1830 ± 235, 1423 ± 200 and 556 ± 50 nm at pH 5.5, 5.8, and 6.2, respectively. Larger particle sizes (>1 μm) at

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pH 5.5 and 5.8 (Figure 8) were due to the formation of large aggregates when the net charge on the protein surface was reduced to near pI. Interaction or complexation with pectin even at 0.15% drastically reduced the particle size ($P < 0.05$), indicating that pectin molecules formed soluble complexes with the proteins and prevented them from extensive aggregation via electrostatic and steric repulsion (Jones et al., 2010b). Interestingly, particle sizes did not change much at higher pectin concentration. At 0.6% pectin, mean diameters of H-complexes were 334, 408, and 247 nm at pH 5.5, 5.8, and 6.2, respectively. Small particle sizes and stability against aggregation of H-complexes, especially those formed at pH 5.5, were due to high electrostatic repulsion from adsorbed pectin polymers (Jones et al., 2009a). These results suggest that adsorption of pectin reached a saturation at 0.45% pectin in H-complexes at pH 5.5 and 5.8 and in the presence of 0.3% pectin in H-complexes at pH 6.2 with no further decrease in zeta-potential at higher pectin concentration. In this study, we selected heated mixed of 3% WPI with 0, 0.3 and 0.45% pectin formed at pH 5.5, 5.8 and 6.2 in determining the optimum condition for emulsifying and stabilizing o/w emulsion containing 20%.
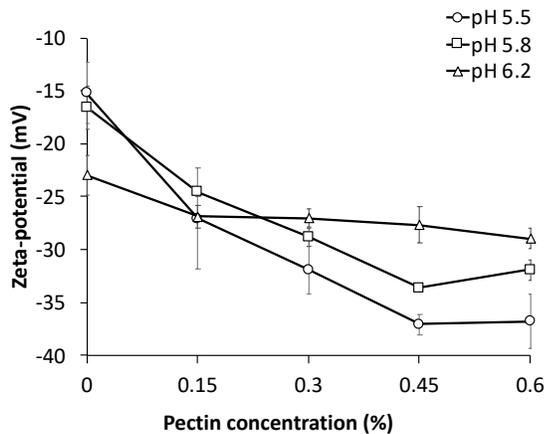

Figure 7 ζ- potential of H-complexes formed at difference pH and pectin concentration
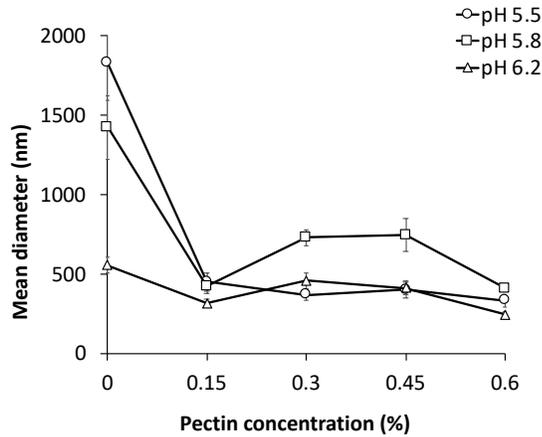
Figure 8 Mean particle size of H-complexes formed at difference pH and pectin concentration

4.4.2 Emulsion

4.4.2.1 Mean diameter and Zeta potential of oil droplets

Heated 3% (w/w) WPI with 0, 0.3 and 0.45% pectin complexes were diluted before emulsification such that the final emulsions contained 2% (w/w) WPI with 0, 0.2 and 0.3% pectin, respectively. The droplet mean diameters (Table 2) and size distributions (Figure 9) were used to report the emulsion droplet sizes. Without pectin, the average droplet diameters (d_{43}) of emulsions stabilized by H-WPI at pH 5.5, 5.8 and 6.2 were 35.7 ± 14.3, 40.2 ± 11.2 and 59.3 ± 8.5 μm, respectively. The size distributions showed broad peak in all three emulsions with the broadest distribution from H-WPI formed at pH 6.2 (Figure 9c). Broad peak characteristic suggested nonhomogeneous droplet sizes. When the emulsions were acidified to pH 5.0 extensive flocculation and coalescence occurred due to the lack of sufficient net charge at near pI (Surh et al., 2006, McClements, 2007). As shown in Table 3, in the absence of pectin, emulsion droplets carried net negative charge with the zeta potentials ranging from -19.8 ± 4.7 to -27.2 ± 2.9 mV. The absolute zeta potentials were less

than 30 mV which is required for sufficient stabilization.

Mean droplet sizes (d_{43}) of H-complexes-stabilized emulsions drastically decreased ($P < 0.05$) when H-complexes contained 0.3% pectin (e.g., 0.2% in the final emulsion). The smallest mean droplet size was observed in emulsions stabilized by H-complexes formed at pH 5.8. Increasing pectin concentration in the H-complexes resulted in further reduction in d_{43} in emulsions stabilized by H-complexes formed at pH 5.5 and 6.2 but the size remained unchanged with H-complexes formed at pH 5.8. The zeta potentials of H-complexes emulsions significantly decreased to < -35 mV. As shown previously, H-complexes were more negatively charged compared to H-WPI. The adsorption of H-complexes on the droplet surface contributed to the higher charge potential. In addition, the adsorption of excess pectin could be enhanced due to increased positively charged of protein on the droplet surface during pH adjustment (Guzey et al., 2004). The combined electrostatic and steric repulsion resulted in a decrease in mean droplet sizes. However, when the concentration of pectin in emulsions increased from 0.2 to 0.3%, the electrical charge remained unchanged. This indicated that adsorption of H-complexes and excess pectin on the droplet interface was saturated at low pectin concentration, thus the high repulsion prevented the additional pectin to adsorb at the interface (Salminen and Weiss, 2014).

It should be noted that, at 0.3% pectin, emulsions prepared by H-complexes at pH 5.8 exhibited a unimodal distribution with the smallest mean droplet size (Figure 9b), suggesting that the emulsion was homogenous with similar droplet sizes (McClements, 2007). H-complexes at pH 5.5 formed emulsions having bimodal distribution with the

major peak centered around 7 μm and the minor peak centered around 1 μm (Figure 9a).

The small population of small droplets in the emulsions may be due the sufficient amount of

H-complexes coated at the droplet surface whereas large droplets may be due to

coalescence.   Inability of the H-complexes to form thick adsorbed membrane could lead to

coalescence (Surh et al., 2006). Emulsions prepared by H-complexes formed at pH 6.2

showed the broadest size distribution (Figure 9c) in addition to having the largest droplet

sizes. As described above, H-complexes at pH 6.2 were the least negatively charged

compared to H-complexes formed at pH 5.5 and 5.8. The lower charge potential could not

prevent flocculation and coalescence during emulsification, resulting in the emulsions

having very large droplet sizes with high polydispersity (Cho and McClements, 2009). The

excess pectin, however, could adsorb at the droplet interface after the emulsion was formed

such that the zeta potentials of these emulsions were not different from those formed by H-

complexes at pH 5.5 and 5.8.

It is interesting to note that emulsions stabilized by H-complexes at pH 5.8 showed

good stability during pH adjustment to near pI (< 3 μm). We speculate that the larger

aggregate sizes of H-complexes at pH 5.8 (> 500 nm.), compared to those formed at pH 5.5

and 6.2 at any pectin concentration, could form thicker layer at the droplet surface which

sufficiently prevent the extensive aggregation in emulsion.

Table 3 Mean droplet diameter (d43) and zeta-potential of H-complexes stabilized in emulsions with different pectin concentration

pH of H-complexes	Pectin (%)	d_{43} (μm $\pm$ SD)	Zeta-potential (mV $\pm$ SD)
5.5	0	35.7 ± 14.3^a	-27.2 ± 2.9^a
	0.2	15.0 ± 4.0^a	-41 ± 1.4^b
	0.3	5.2 ± 1.2^a	-40.1 ± 2.8^b
5.8	0	40.2 ± 11.2^a	-24.1 ± 2.3^a
	0.2	2.8 ± 0.6^b	-38.3 ± 3.3^b
	0.3	1.6 ± 0.2^b	-38.4 ± 3.0^b
6.2	0	59.3 ± 8.5^a	-19.8 ± 4.7^a
	0.2	26.5 ± 4.6^b	-40.9 ± 1.9^b
	0.3	17.5 ± 3.8^b	-39.1 ± 3.0^b

[a,b]*Different letters at the same pH during complex formation in emulsion indicate significant differences (P < 0.05).*
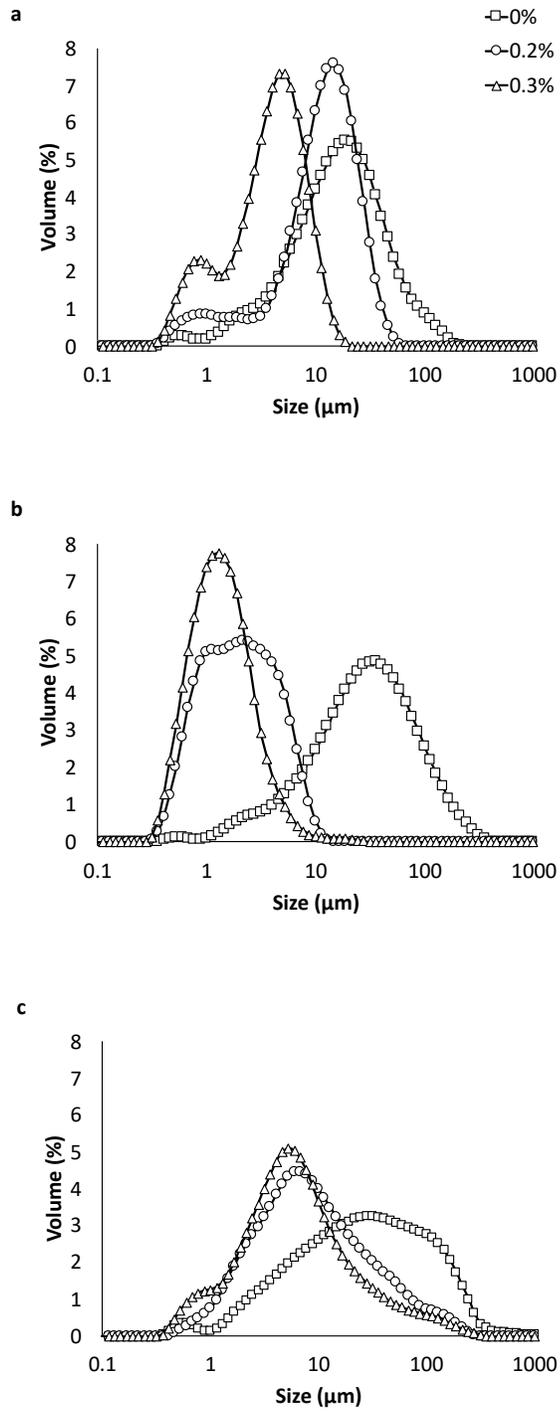
Figure 9 Droplet size distribution of emulsions stabilized by H-complexes formed at pH 5.5 (a), pH 5.8 (b) and pH 6.2 (c) with difference pectin concentration

4.4.2.2 Rheological properties of emulsions

Rheological properties of emulsions were measured immediately after emulsions were prepared. Plots of apparent viscosity versus shear rate are shown in Figure 10. Rheological properties as described by the consistency coefficient (K) and flow behavior index (n) were determined using the Power Law model (Table 4). All emulsions stabilized by heated WPI without pectin exhibited a shear-thinning behavior with a deflection point at certain shear rates (Figure 10). This might be due to the disruption of flocs at these shear rates, thus decreasing the effective volume fraction and lowering the viscosity with further increasing shear rate (Franco et al., 1995, Surh et al., 2006, Huan et al., 2016c). Among H-WPI-stabilized emulsions, H-WPI at pH 6.2 formed the most viscous emulsion which reflected the size property.

The deflection point was not observed when H-complexes were used to prepare the emulsions. Flow behavior of emulsions prepared by H-complexes ranged from 0.256 to 0.566, suggesting a shear-thinning behavior (n < 1) (Huan et al., 2016a). Among emulsions stabilized by H-complexes formed at the same pH, the effect of pectin concentration was not observed ($P > 0.05$). The effect of pH during H-complexes formation, however, was apparent. Flow behavior of emulsions stabilized by H-complexes formed at pH 5.8 revealed lower viscosity compared those to stabilized by H-WPI, reflecting their smaller droplet sizes and no bridging or depletion flocculation. Emulsions prepared by H-complexes at pH 5.5 had similar n but higher K values compared to those formed by H-complexes at pH 5.8 ($P > 0.05$).

H-complexes at pH 6.2 formed the emulsions with the lowest n and the highest K values ($P < 0.05$). As shown above, these samples exhibited very large droplet sizes with high degree of polydispersity which could explain their high viscosity and highly shear thinning behavior (McClements, 2007). Emulsions having higher pectin concentration had slightly higher K value, suggesting that depletion flocculation due to non-adsorbed pectin could contribute to its rheological properties (Chanamai and McClements, 2001).

Table 4 Power law model parameters for emulsions stabilized by H-complexes with different pectin concentration

pH of H-complexes	Pectin (%)	K (Pa·s^n)	n
5.5	0	0.141^a	0.565^a
	0.2	0.378^{ab}	0.552^a
	0.3	0.722^b	0.455^a
5.8	0	0.277^a	0.519^a
	0.2	0.155^b	0.510^a
	0.3	0.200^b	0.566^a
6.2	0	0.412^a	0.445^a
	0.2	1.500^a	0.258^a
	0.3	1.810^a	0.256^a

[a,b]*Different letters within each pectin concentration in emulsion indicate significant differences (P < 0.05).*
Consistency coefficient (K) and flow behavior index (n) were determined by fitting flow curves to the Power Law model. The data were the average from 3 measurements
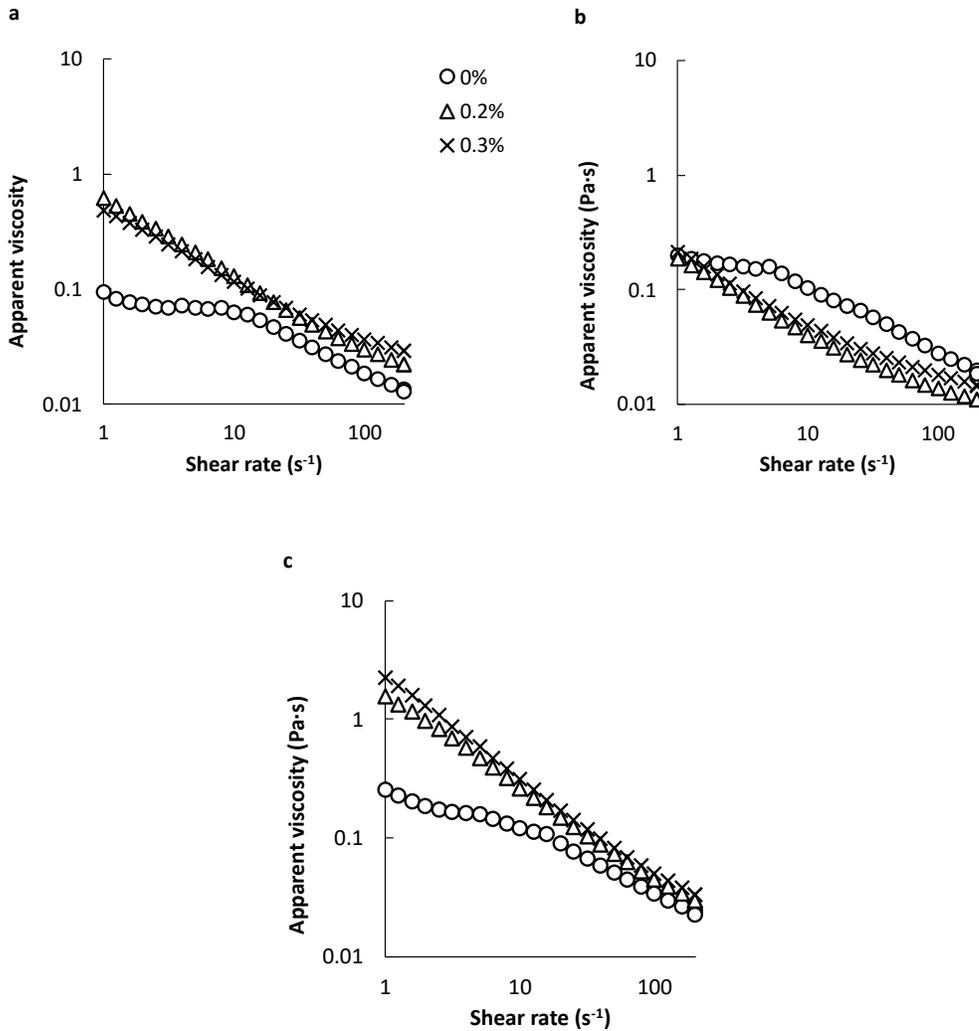
Figure 10 Apparent viscosity of fresh emulsions prepared with H-complexes formed at pH 5.5 (a), pH 5.8 (b) and pH 6.2 (c) with different pectin concentration

4.4.2.3 Creaming stability of emulsions

Stability against creaming was monitored by measuring the creaming index (Figure 11). Emulsions stabilized by heated whey protein at any pH were unstable and separated into two layers within 24 h (data not shown). A visible serum phase appeared rapidly due to the large droplet sizes and the inability of the protein to provide sufficient electrostatic and steric stabilization. For emulsion stabilized by H-complexes with 0.2% pectin, all samples

showed significant reduction in creaming index, but 1.5% and 2.5% creaming were observed on Day 7 in those stabilized by H-complexes at pH 5.5 and 6.2, respectively. Only emulsions prepared by H-complexes formed at pH 5.8 were stable for 7 days but 2.2% creaming was observed on Day 14. The most stable (> 30 days) emulsions were achieved from H-complexes formed with higher pectin at pH 5.5 and 5.8, while H-complexes formed at pH 6.2 did not lead to stable emulsion. This could be due to the smaller droplet sizes with higher surface charge in samples stabilized by H-complexes at pH 5.5 and 5.8 compared to those stabilized by H-complexes at pH 6.2. It should be noted that though emulsions made by H-complexes at pH 6.2 exhibited much higher viscosity they were not stable. On the other hand, emulsions formed with H-complexes at pH 5.5 and especially 5.8 were stable despite their lower viscosity. These results suggest that, with limited interaction/complexation with pectin, the H-complexes heated at pH 6.2 did not exhibit good emulsification properties as shown by larger droplet sizes. The excess pectin (non-interacting) could lead to destabilization (Gu et al., 2004). More complexation between WPI and pectin when the H-complexes were formed at pH 5.5 and 5.8 allowed the adsorption of pectin on the oil droplets during emulsification, thus increased high electrically charge which was able to stabilize the emulsions.
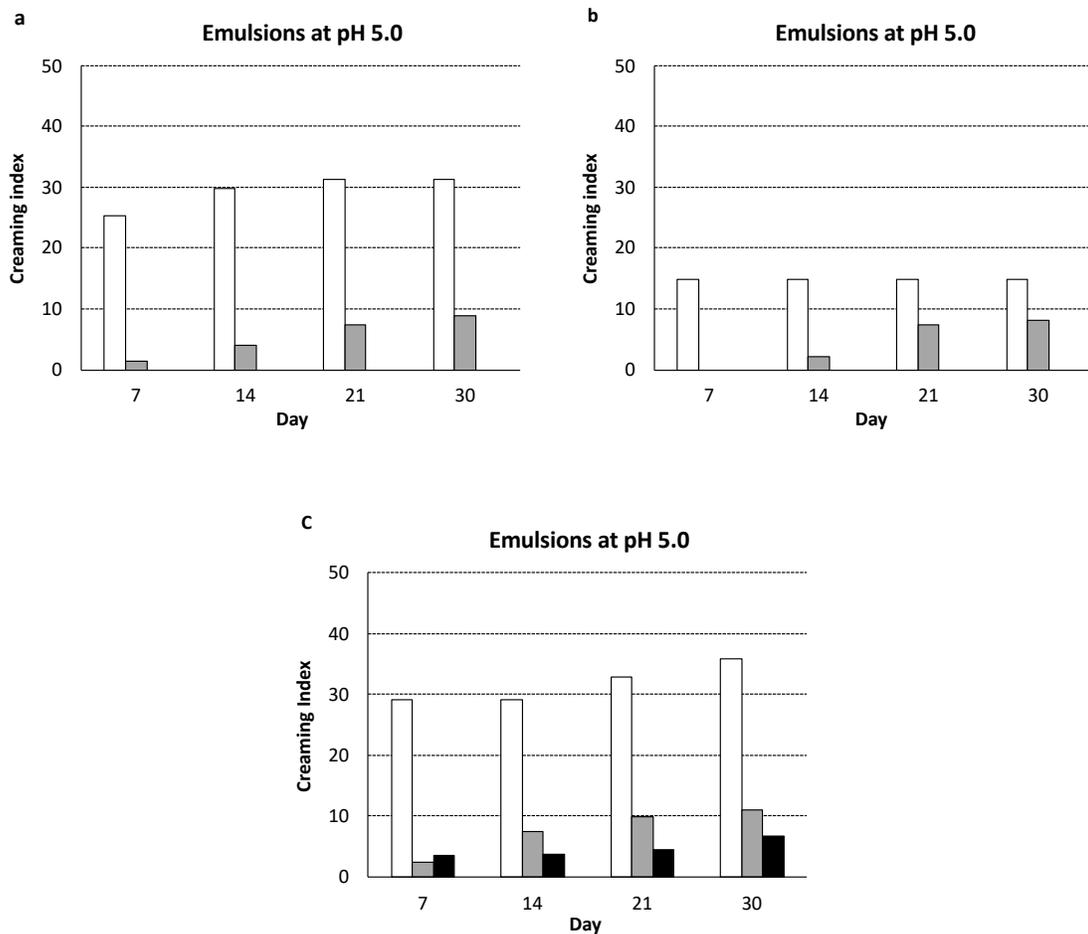
Figure 11 Creaming stability of emulsions prepared with H-complexes formed at pH 5.5 (a), pH 5.8 (b) and pH 6.2 (c) with different pectin concentration; 0% (white), 0.05% (grey) and 0.15% pectin (black). Emulsions were stored for 30 d at room temperature

4.4.3 Conclusion

Pectin concentration and pH during the formation of H-complexes played significant roles in emulsification properties and stability of emulsions containing 20% oil. The presence of H-complexes formed at pH 5.8 and 5.5 could protect the emulsions from acid-induced destabilization while stability was not achieved with H-complexes formed at pH 6.2. Interaction between WPI and pectin during complex formation allowed the H-

complexes to adsorb at the droplet interface as well as prevent flocculation and coalescence.

The properties of the emulsions can be correlated to the corresponding H-complexes

properties. H-complexes formation condition can be optimized allowing the H-complexes to

be used as clean-label emulsifier and stabilizer in foods containing higher oil content.

CHAPTER 5 CONCLUSIONS

5.1 Concluding Statements

The overall goal of this thesis was to improve emulsification properties of whey protein by forming soluble complexes with pectin. The effect of heating temperature, pectin concentration and pH were investigated. Complexation between WPI and pectin led to increased negatively charged and altered aggregate sizes which improved emulsification properties of the proteins. Our results clearly showed that pectin concentration, heating temperature and heating pH influenced the physical and emulsification properties of the complexes. Heated complexes formed at 85°C formed the emulsions that were most stable against creaming and heating. Complex formation pH also played a major role in the stability and rheological properties of the emulsions containing higher oil content. Thus, heated WPI-pectin complexes can be optimized for different applications.

5.2 Applications and Future research

Heated WPI-pectin soluble complexes could be utilized as emulsifiers and stabilizers in the environment where the functional properties of the protein are limited, particularly in clean label foods and beverages. Future study may focus on testing the heated complexes in commercial food formulations since other ingredients and processing may influence colloidal interactions and thus the properties of the final products. In addition, future study may investigate the utilization of these complexes after spray drying in order to evaluate their potential as commercial food ingredients.
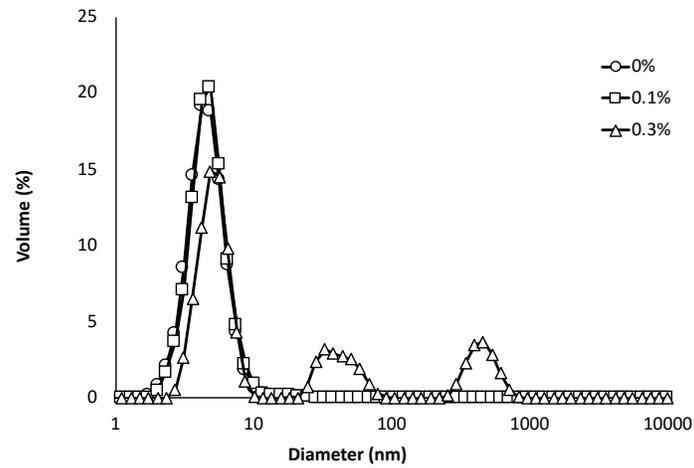
*Figure 12 Size distribution of WPI with different pectin concentration*
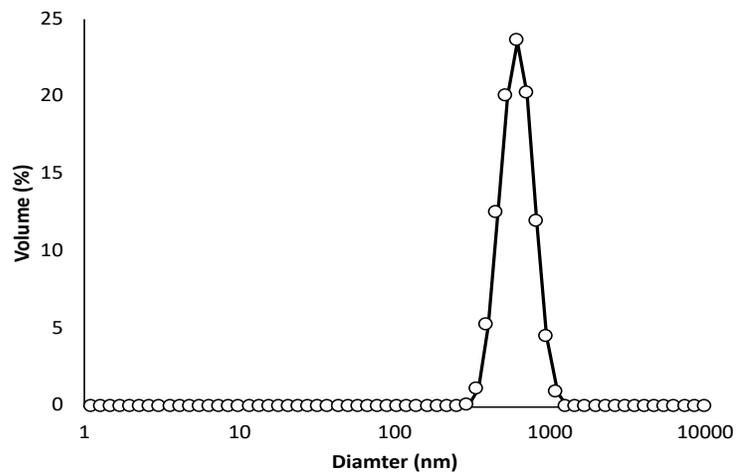

Figure 13 Size distribution of pectin at 0.3% concentration

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