APPLICATION OF SOLUBLE WHEY PROTEIN-CARBOXYMETHYLCELLULOSE COMPLEX IN EMULSION AND ACID-INDUCED GELATION

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

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APPLICATION OF SOLUBLE WHEY PROTEIN-CARBOXYMETHYLCELLULOSE COMPLEX IN EMULSION AND ACID-INDUCED GELATION

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ABSTRACT

Soluble complex between whey protein isolate (WPI) and carboxymethylcellulose (CMC) can be formed at pH above the isoelectric point of the protein. This complex can be utilized to enhance functional properties of the biopolymers and thus improve texture and stability of many food products. This study investigated the effect of molecular weight and concentration of CMC on emulsion stabilization and acid-induced gelation.

In the first study, the influence of CMC concentration and molecular weight (M_w = 270k, 750k, and 2500kDa) on the stability and properties of WPI/CMC-stabilized oil-in-water emulsions was investigated. Emulsions were prepared using soluble WPI-CMC complexes by homogenization 5% vegetable oil with 95% mixed WPI-CMC solution (0.5% WPI and 0-0.5% CMC, pH 7.0) at 12,000 rpm for 1 min, followed by sonication at 30% amplitude of total power for 5 min, and the pH was adjusted to 5.2. Emulsions were assessed by measuring ζ-potential, droplet size, creaming stability, rheological properties, and protein surface coverage. In the absence of CMC, the WPI-stabilized emulsions were unstable to droplet flocculation and

coalescence due to the relatively low droplet charge. ζ-potential and droplet size indicated that WPI-CMC complexes adsorbed to the droplet surfaces and thus reduced droplet flocculation and coalescence. Both CMC M_w and concentration significantly influenced the properties and stability of acidified emulsions. At low CMC concentrations, stability was improved due to increased droplet charge and protein surface coverage, while the effect of viscosity dominated at high CMC concentrations. High M_w of CMC contributed to better stability compared to lower M_w CMC. At proper concentration, emulsions containing high M_w CMC (2500k) were the most stable and showed no separation even after 15-day storage. In the second study, acid-induced gelation of heated WPI and CMC soluble complex was investigated. Heated soluble WPI-CMC complexes were prepared by mixing the biopolymers at pH 7 and heated at 85 ^{o}C for 30min. Gels were formed by the addition of glucono-δ-lactone (GDL) and compared to those formed from WPI polymer (protein heated alone) with added CMC. All gels contained 5% protein and 0-0.125% CMC (M_w = 270k, 680k, and 750kDa). Results showed that CMC molecular weight and biopolymer ratio were the major factors affecting gel properties. For 270k and 750k CMC, gels from heated WPI-CMC complex showed improved gel hardness and, at certain CMC concentration, improved water holding capacity. Confocal laser scanning microscopy (CLSM) results revealed that gel structure largely depended on CMC concentration. Overall, gels from heated WPI-CMC complex showed smoother structure and less porosity, indicating less phase separation. Furthermore, gels showed better mechanical properties when heated WPI-CMC complex at higher protein

concentration.

Overall, both unheated and heated WPI-CMC complex improved the emulsification and cold gelation of whey protein. The M_w of CMC significantly affects their interactions with whey protein and thus the functional properties of the complexes. High M_w CMC at optimum concentration resulted in the improvement of emulsion stability and acid-induced gel properties. By utilizing proper M_w and optimum concentration, WPI-CMC complex can be applied as a novel food ingredient.

CHAPTER 1

INTRODUCTION

1.1 Need for the research

Biopolymer interactions are widely used to improve the texture and shelf-life of food products. Many studies on the interactions between protein and polysaccharide have been reported (Pereyra and others 1997; Laneuville and others 2000; Turgeon and others 2003; Neirynck and others 2007; Schmitt and Turgeon 2011; Dickinson 2008). Attractive interactions between protein and polysaccharide can lead to soluble and/or insoluble complexation, cosolubility or segregation (Schmitt and others 1998; Rodríguez Patino and Pilosof 2011). Functional properties of protein are generally improved by complexation with polysaccharides. The formation and solubility of protein-polysaccharide complex mainly depend on pH, ionic strength, biopolymer ratio and concentration. Other factors such as types of biopolymers (charge density, molecular weight, et al.), temperature and pressure also influence the complex formation (Schmitt and Turgeon 2011). Maximum protein-polysaccharide complex formation could be reached at pH values below or around the pI of protein due to the opposite charge carried by these two biopolymers. However, at pH values above the pI of proteins, electrostatic interactions between biopolymers can still occur (Doublier and others 2000; Vardhanabhuti and others 2009; Girard and others 2002a). In this case, any electrostatic interaction involves the anionic polysaccharide

interacting with positively charged local patches on the proteins (Dickinson 1998).

Heating mixed biopolymers at near neutral pH can result in heated soluble complex having different size and shape compared to heated protein aggregates without polysaccharides. When formed at appropriate conditions, heated soluble protein-polysaccharide complex could also offer better functional properties. It has been reported that dextran sulfate improved thermal stability of β-lactoglobulin by altering its aggregation and the complexation between the two by heating at near neutral pH resulted in less turbid solution or gels (Vardhanabhuti and others 2009). Zhang and others (2014) recently demonstrated that heated soluble whey protein/pectin complex at pH 7 resulted in finer gel microstructure with less porosity and smoother network, which significantly improved gel strength and water holding capacity compared to heated whey protein alone with addition of pectin.

Soluble protein and polysaccharide complex has been studied in emulsions. When prepared at pH 6, the mixed caseinate-dextran sulfate solution resulted in a soluble complex rather than a coacervate and the emulsion obtained by this approach gave much stronger interfacial films than the bilayer emulsion, leading to a more stable emulsion system (Jourdain and others 2009). Lutz and others (2009a) reported that once a soluble WPI-pectin complex was formed at pH 6, the resulting emulsion was stable with small droplet size, minimum creaming and low water transport.

Though there has been an increasing interest in the functional properties of unheated and heated soluble protein-polysaccharide complexes at near neutral pH, knowledge in this area is still very limited. Understanding how different factors

(intrinsic and extrinsic) could affect the complex formation and their functional properties can lead to the design of proper complexes for different applications.

1.2 Objectives of the study

The overall objective of this study was to investigate how molecular size (e.g., molecular weight) of CMC affects the formation and functional properties of unheated and heated soluble complexes between WPI and CMC. Specific objectives were: (i) to investigate the effects of molecular weight of CMC on the stability of emulsions stabilized by unheated WPI-CMC complexes, (ii) to characterize the emulsions stabilized by WPI-CMC complexes, (iii) to determine the effects of CMC molecular weight on acid-induced gelation of heated WPI-CMC soluble complexes, and (iv) to determine whether there was a relationship between the molecular properties of the complexes (both heated and unheated) and their functional properties.

CHAPTER 2

LITERATURE REVIEW

2.1 Whey protein

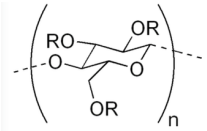
Whey protein is a mixture of globular proteins isolated from whey, a by-product of cheese production. It represents 20% of the total protein content of cow's milk. It contains four major proteins, namely β-lactoglobulin (~ 65%), α-lactalbumin (~ 25%), bovine serum albumin (~ 8%), and immunoglobulins. Whey protein typically comes in three major forms: whey protein concentrate (WHC), whey protein isolate (WPI), and whey protein hydrolysate (WPH).

Whey proteins have been widely used in food industry due to its high nutritional values and unique functional properties. It has been reported that whey proteins are utilized in many different applications and the effects on bone, muscle, blood, brain, immune, infection and metabolism have attracted lots of interest. Its effect on reducing the risks of diseases such as heart disease, cancer and diabetes is currently being investigated (Krissansen 2007). The increased use of whey protein in food industry is due to its excellent thermal stability, gelation, foaming and emulsification properties (Foegeding and others 2002). Whey proteins are well known as replacements for egg proteins in confectionery and bakery products, and are also used as functional ingredients and as milk replacers in dairy products such as ice cream (de Wit 1998). Whey proteins form interfacial films that stabilize emulsion and foams such as ice cream, salad dressings, and etc. (Ruger and others 2002;

Akalın and others 2007; Turgeon and others 1996). Gelation properties of whey protein has been utilized to improve texture and water holding capacity of meat and yogurt (Zhang and others 2014; Lyons and others 1999). The recent increased application of whey protein has been for nutritional beverages (Vardhanabhuti and Foegeding 2008; Keowmaneechai and McClements 2006).

2.2 Carboxymethylcellulose (CMC)

Carboxymethylcellulose (CMC) or cellulose gum, is derived from cellulose, which is made water-soluble by a chemical reaction. The solubility is achieved by introducing carboxymethyl groups along the cellulose chain, which makes hydration of the molecule possible. It is often used as its sodium salt, sodium carboxymethylcellulose.



R = H or CH_2CO_2H

CMC has the ability to impart viscosity to aqueous solutions. CMC is pseudoplastic by nature and can show thixotropic and non-thixotropic rheology. The viscosity is proportional to the average chain length of the CMC molecule or the degree of polymerization (DP). The average chain length and the degree of substitution (DS) determine the molecular weight of CMC. The viscosity of CMC increases rapidly with increasing degree of polymerization.

CMC is used in food industry as a viscosity modifier or thickener, and to stabilize emulsions and improve foaming properties in various products (Hansen and

Black 1972; Mann and Malik 1996; Girard and others 2002b). It is also used extensively in gluten free and reduced fat food products (Chillo and others 2007). It has been reported that the long-term stability of acidified milk drinks with high molecular weight CMC was better than that with low molecular weight CMC (Du and others 2009). Capitani and others (2007) evaluated the thermostability of β-lactoglobulin and α-lactalbumin by complexation with CMC and both complexes showed a maximum stability at pH 4 due to electrostatic interactions between proteins and CMC. Besides controlling the rheological properties, CMC is known for its excellent water retaining capacity.

2.3 Protein-polysaccharide interactions

Proteins and polysaccharides are present together in many kinds of food systems, and contribute to the structure, texture and stability of food (Doublier and others 2000). Interactions with polysaccharides will influence the functional properties of food proteins, such as solubility, heat stability, gel formation, emulsification and foaming properties (Ye 2008). Control and manipulation of protein-polysaccharide interactions is a key factor in the development of novel food processes and products. Interactions between proteins and polysaccharides are mainly driven by electrostatic interactions, which can divide the mixed biopolymers into three groups: co-solubility, incompatibility, and complexation. A dilute non-interacting biopolymer mixture of proteins and polysaccharides may be co-soluble, forming a stable solution. If protein and polysaccharide carry opposite charges (e.g., pH < pI), electrostatic attraction

between the biopolymers will lead to the formation of soluble complex/coacervates or associative phase separation. However, if proteins and polysaccharides repel each other (e.g., pH > pI), mixtures of biopolymers are often incompatible, leading to thermodynamic incompatibility (segregation). Phase separation results in one phase rich in proteins and the other phase rich in polysaccharides (Tolstoguzov 1991).

2.3.1 Protein-polysaccharide complexes

Complexes can be regarded as a new type of food biopolymers, the functional properties of which showed markedly differences from those of macromolecular reactants (Doublier and others 2000). The formation of an electrostatic complex is usually a reversible process, depending on parameters such as pH and ionic strength. Generally, electrostatic complexes dissociate with ionic strength exceeds 0.2-0.3 M, or when the pH is above the pI of the protein (Ye 2008). Complex formation between proteins and polysaccharides usually occurs at pH values below the isoelectric point of the proteins and at low ionic strength. The interactions between the two biopolymers reach the maximum when proteins carry a net positive charge and behave as polycations at pH values below the pI, while polysaccharides have a net negative charge and behave as polyanions at pH ranging between the pK and the pI (de Kruif and others 2004). However, electrostatic interactions between proteins and polysaccharides can still occur at near neutral pH. It is due to the fact that anionic polysaccharides can interact with positively charged patches on the proteins (Dickinson 1998; Doublier and others 2000; Tolstoguzov 1997). Recent studies have

indicated that this type of complex showed enhance functional properties such as heat stability and acid-induced gelation.

2.3.2 Factors influence the complexation

The formation of complexes is primarily influenced by pH, ionic strength, biopolymer concentration and protein to polysaccharide ratio. Some other factors such as the biopolymer charge density and molecular weight, temperature, pressure or stirring have been shown to also influence complexes formed at pH < pI (Schmitt and Turgeon 2011). These parameters have been extensively investigated in references and discussed in details for many protein/polysaccharide pairs.

pH. pH plays an important role in the formation of protein-polysaccharide complexes because it influences the net charge carried by the biopolymers. At pH values below pI of the protein, an anionic polysaccharide and a protein carries opposite net charges, resulting in a maximum electrostatic interaction. It has been reported that complex formation of β-lactoglobulin/acacia gum increased when pH of the mixture decreased from 5.0 to 3.6 due to increased positive net charge on the protein (Schmitt and others 1999). A similar effect was reported when gum arabic was mixed with pea protein isolate, and complexes were obtained at pH 3.2 to 4.0 (Liu and others 2010). At pH values above the pI, e.g. at near neutral pH, electrostatic interaction can still occur due to the interaction between anionic polysaccharide with positively charged subunits on the proteins, forming soluble interbiopolymer complexes (Doublier and others 2000; Vardhanabhuti and others

2009; Girard and others 2002a).

Ionic strength. The net charge on proteins and polysaccharides is reduced by interaction with salts, leading to a decrease in the electrostatic attraction between the biopolymer molecules. At high ionic strength, salt would screen the charges of the protein and polysaccharides, which affects the formation of complexes (Ye and others 2000; Vardhanabhuti and Foegeding 2008; Weinbreck and others 2003a; Galazka and others 1999).

Biopolymer ratio and biopolymer concentration. The protein to polysaccharide ratio will obviously have effect on the charge balance of the mixture, hence influencing complexation. At a certain condition of pH and ionic strength, maximum complexation is obtained at a specific protein to polysaccharide ratio. It was clearly shown that the optimum coacervation was obtained for β-lactoglobulin/acacia gum mixing ratio of 2:1 at pH 4.2 (Schmitt and others 1999). Similarly, Ducel and others (2004) found that optimum coacervation was obtained at pea globulins to arabic gum ratio of 3:7 and pH 2.7, and with α-gliadins to arabic gum ratio of 1:1 at pH 3. Weinbreck and others (2003a) explained that increasing the biopolymer concentration would screen the charges of the biopolymers due to release of more counterions in the solution, thus increase the solubility of the complexes. Furthermore, at high biopolymer concentrations, the mixture will show phase separation which is attributed to the competition between the molecules for the solvent (Tolstoguzov 1997).

Other factors. Processing factors, including temperature, heating time, pressure,

and shear rate can affect the formation and stability of protein-polysaccharide complexes (Laneuville and others 2000; Li and others 2006; Leng and Turgeon 2007). These parameters may induce conformational changes on proteins and polysaccharides such that have influence on the interactions between the two biopolymers. For instance, an increase in temperature enhances hydrophobic interactions and covalent bonding while low temperature is conductive to hydrogen bonding. Globular proteins will be unfolded and denatured at high temperature, exposing more reactive sites for interacting with polysaccharides (Ye 2008). Galazka and others (1999) pointed out that complexation with carrageenan protected the BSA against loss of its functionality due to disulfide bridge formation during or after high pressure treatment.

2.4 Emulsification

Food emulsions are complex in composition. The droplets are stabilized by proteins, small-molecule surfactants, and in certain cases, polysaccharides (Dickinson 2010). Basically, emulsions could be stabilized either by protein-polysaccharide complexes/coacervates or by using the so-called layer-by-layer technique, leading to mixed emulsions or bilayer emulsions. The layer-by-layer technique is most commonly used in food industry even if the two types of emulsions have different properties (Jourdain and others 2009). However, in Jourdain and others (2009), mixed sodium caseinate/dextran sulfate emulsions were much more stable against bridging flocculation even at very low pH compared to

bilayer emulsions due to the different structure of the composite biopolymer at the interface. Complexes between whey protein isolate and HM-pectin were also used for emulsion stabilization at pH 5.5 due to strong electrostatic repulsion between oil droplets (Neirynck and others 2007). Lutz and others (2009b) reported that such complexes were shown to stabilize the external interface of W/O/W emulsions prepared at pH≤6.0. In addition, whey protein/CMC complex-stabilized emulsions showed freeze stability upon storage at -15 ^{o}C for 7 days (Koupantsis and Kiosseoglou 2009). Several other protein/polysaccharide pairs were used to produce acid stable emulsions and could be applied in the beverage industry (Du and others 2009; Klein and others 2010). Furthermore, complex-stabilized emulsions were used for entrapment of flavors and delivery in food matrices (Weinbreck and others 2004; Relkin and others 2004).

2.5 Gelation

Gelation is one of the most important functional properties of proteins and it is widely applied to meat, yogurt, cheese and other gel-based food products (Sodini and others 2005; Everett and McLeod 2005; Lyons and others 1999).

Gelation can be induced by chemical and enzymatic methods, heat treatment or so called cold-set process. Cold-set gelation is a two-step gelation process conducted at ambient temperature. In the first step, protein polymers are obtained by heating the protein solution at below critical gelation concentration, pH above or below the isoelectric point, and low ionic strength. In the second step, acid or salt is added to

the protein polymers to induce gelation by reducing the electrostatic repulsion between the protein aggregates (Bryant and McClements 2000b; Ju and Kilara 1998; de Jong and others 2009; Kuhn and others 2010; Hongsprabhas and Barbut 1997b; de Faria and others 2013). Salt type and concentration have effects on gel properties. Bryant and McClements (2000c) found that adding 100-400 mM NaCl to whey protein polymer solutions resulted in gels with increased turbidity, shear modulus (G*) and elasticity. When compared with gels induced by addition of NaCl, those containing $CaCl_2$ were more rigid due to calcium being more effective at screening charges and its ability to form Ca^{2+} bridges between protein molecules (Bryant and McClements 2000b; Vardhanabhuti and others 2001). Cold-set gelation can also be induced by adding gluconic-δ-lactone, which gradually reduces the pH of protein polymers and thus forming the gel. During the acidification process, additional disulfide bonds are formed between aggregates to strengthen the gel network (Alting and others 2000).

Polysaccharides are added to alter and improve the properties of acid-induced protein gels. de Jong and van de Velde (2007) investigated the effect of charge density of polysaccharide on the microstructure and physical properties of acid-induced WPI gels, and pointed out that charge density was the dominant factor that determine the micro-phase separation. In WPC-gellan systems, an increase in gel mechanical properties was observed due to the formation of electrostatic complexes between the proteins and polysaccharides at pH 4 (Picone and da Cunha 2010). Zhang and others (2014) pointed out that the water holding capacity (WHC)

of acid-induced whey protein gels depended on the concentration of pectin. Addition of pectin at low concentration had little effect on WHC while high pectin concentration had sever adverse effect on WHC due to thermodynamic incompatibility between protein and pectin.

Interactions between proteins and polysaccharides have been extensively utilized in the food industry due to their enhanced properties compared to those of protein alone. Most studies, however, have been conducted on the mixtures at pH < pI, while investigations of complexes formed at pH > pI have been limited. It has been shown that unheated and heated soluble complexes between protein and polysaccharide at pH > pI have a great potential to be novel food ingredients due to their improved functional properties. Understanding different factors that affect their formation and functional properties could lead to the design of the complexes that are suitable for different applications.

CHAPTER 3

THE EMULSIFICATION PROPERTIES AND STABILITY OF WHEY PROTEIN-CMC COMPLEX

Manuscript to be Submitted for Publication

3.1 Abstract

The influence of CMC concentration and molecular weight (M_w = 270k, 750k, and 2,500kDa) on the stability and properties of WPI/CMC-stabilized oil-in-water emulsions was assessed by measuring ζ-potential, droplet size, creaming stability, apparent viscosity and protein surface coverage. Emulsions were prepared with soluble WPI-CMC complex by ultrasonicating 5% oil, 0.5% WPI and 0-0.5% CMC at pH 7. After emulsification, pH was adjusted to 5.2. In the absence of CMC, the WPI emulsions were unstable to droplet flocculation and coalescence due to the relatively low droplet charge. ζ-potential and droplet size measurements indicated that WPI-CMC complex absorbed to the droplet surfaces and thus reduced droplet flocculation and coalescence. The emulsion with 0.08% CMC 2,500k showed the most stable properties. Although the acidic emulsions containing 0.5% CMC 2,500k remained stable within 10 days after preparation due to the high viscosity and/or weak gel-like network, it showed separation for further storage. Both CMC concentration and M_w influenced the stability of acidified emulsions, and high M_w of CMC at proper concentration contributed to the long-term stability of emulsion

system.

3.2 Introduction

Biopolymer interactions are widely used to improve the texture and shelf-life of food products. Many studies on the interactions between protein and polysaccharide have been reported (Pereyra and others 1997; Laneuville and others 2000; Turgeon and others 2003; Neirynck and others 2007; Schmitt and Turgeon 2011; Dickinson 2008). Functional properties of protein are generally improved by complexation with other polysaccharides. The formation and solubility of protein-polysaccharide complex mainly depend on pH, ionic strength, biopolymer ratio and concentration. Other factors such as types of biopolymers (charge density, molecular weight, etc.), temperature and pressure also influence the complex formation (Schmitt and Turgeon 2011). Protein-polysaccharide complex formation reaches the maximum at pH values below or around the pI of protein due to the opposite charge carried by these two biopolymers. However, at pH values above the pI of proteins, e.g. at neutral pH, electrostatic interactions between biopolymers can still occur (Doublier and others 2000; Vardhanabhuti and others 2009; Girard and others 2002a). In this case, any electrostatic interaction involves the anionic polysaccharide interacting with positively charged local patches on the proteins (Dickinson 1998).

Two alternative procedures can be used for stabilization of oil droplets by protein-polysaccharide electrostatic complex: 'bilayer emulsion' preparation and 'mixed emulsion' preparation (Jourdain and others 2008). The so-called

layer-by-layer technique is used for the preparation of bilayer emulsions whereby polysaccharide is added to a protein-stabilized system. Mixed emulsions are prepared by soluble protein-polysaccharide complexes which adsorb onto the oil droplet surface directly. (Jourdain and others 2009). When prepared at near neutral pH, the mixed emulsion approach would show more stable properties compared with the bilayer emulsion. It has been demonstrated that when prepared at pH 6, the mixed caseinate-dextran sulfate solution resulted in a soluble complex rather than a coacervate and the emulsion obtained by this approach gave much stronger interfacial films than the bilayer emulsion, leading to a more stable behavior of emulsion system (Jourdain and others 2009). (Lutz and others 2009a) reported that once a soluble WPI-pectin complex was formed at pH 6, the resulting emulsion was stable with small droplet size, minimum creaming and low water transport.

Polysaccharide plays an important role in a protein-stabilized emulsion and has a great influence on the emulsion properties (Dickinson 2003). Various research have been reported on the addition of polysaccharide to enhance the properties and stability of protein-stabilized oil-in-water emulsions (Ye and others 2000; Surh and others 2006; Long and others 2013; Dickinson and Pawlowsky 1997; Neirynck and others 2007). Although the stabilization of emulsion is primarily dependent on the concentration of polysaccharide, molecular properties of polysaccharides can significantly influence emulsification properties. It has been investigated that whey protein-stabilized emulsion with low molecular weight and low degree of deacetylation of chitosan was less stable due to the loss of interfacial coadsorption

efficiency and interfacial net charge (Laplante and others 2005b). Du and others (2009) reported that CMC with high molecular weight and high degree of substitution increased the viscosity of the solutions and increased the electrostatic repulsion between casein particles, respectively, leading to the long-term stability of acidified skim milk drinks.

Whey protein isolate (WPI) is one of the most used proteins in food emulsions due to its excellent surface activity. The emulsification properties of WPI with different polysaccharides have been widely studied (Girard and others 2002c; Singh and others 2003; Laplante and others 2005a; Sun and others 2007; Klein and others 2010; Li and others 2012a). As one of the most important derivatives of cellulose, carboxymethylcellulose (CMC) is an anionic water soluble polysaccharide and has been used in a wide range of food products (Nussinovitch 1997). It has been demonstrated that WPI/CMC network of complex formed at pH 4.2 was more effective in protecting oil droplets against coalescence than emulsion containing WPI alone due to higher protein load (Girard and others 2002b). WPI had weak interfacial activity while the use of complex as emulsifier made it possible to adsorb larger amounts of protein that would be involved in the network formation between complex. Koupantsis and Kiosseoglou (2009) reported that by complexation of whey protein with CMC at pH value below pI, interactions might take place at lower pH, leading to the stability of whey protein-stabilized emulsion during ageing, heating or freezing. To our best knowledge, the emulsification properties of WPI-CMC complex formed at neutral pH and the effect of CMC molecular weight on

WPI-stabilized emulsion have not been studies.

The objective of our study was to investigate the emulsification properties of mixed emulsion formed by first preparing a bulk aqueous solution of WPI-CMC complex at pH 7.0, and using the complex as the emulsifying agent during subsequent homogenization. After emulsification, the pH was adjusted to 5.2. The reason for choosing pH 5.2 in this study was that it is the pH value near pI of whey protein. At this pH, emulsion prepared with whey protein alone is unstable due to the least electrostatic repulsion between droplets. Formation of WPI-CMC complex was expected to improve the stability of whey protein-coated emulsion. We aimed to investigate the influence of CMC molecular weight and concentration on the interactions between CMC and WPI at pH value near pI and thus on the emulsification properties and stability of emulsion system. This study can provide a better understanding of protein and polysaccharide interactions and the outcomes could be applied in emulsion food products having pH values near pI of the protein.

3.3 Materials and methods

3.3.1 Materials

Whey protein isolate (WPI) Bipro^{TM} was kindly provided by Davisco Foods International Inc. (Le Sueur, MN). According to the manufacturer, WPI contained 97.9% protein and 1.8% ashes on a dry basis. CMC with the average molecular weight (M_w) of 270k, 750k, and 2,500kDa were kindly provided by CP Kelco Inc.

(Atlanta, GA). Phosphate buffer solutions (5mM, pH 7 and 5.2) were made with Milli-Q water (>18.2 MΩ). Commercial vegetable oil was purchased from local supermarket, and all the other reagents were of analytical grade. All ingredients were used without further purification and without correction for their moisture content.

3.3.2 Preparation of stock solutions

WPI stock solution (10%, w/w) was prepared by slowly dissolving protein powder into 5 mM phosphate buffer at pH 7 and kept stirring at room temperature for at least 2 h. CMC stock solution (1%, w/w) was prepared by slow addition of CMC powder into phosphate buffer at pH 7 and at 85 ^{o}C for 1 h under continuous stirring. After heating, the CMC stock solution was cooled in ambient temperature before weight adjustment to bring the concentration back to 1%. The two stock solutions were stored at 4 ^{o}C in the refrigerator overnight for complete hydration.

3.3.3 Preparation of emulsions

All oil-in-water emulsions containing 5% of oil, 0.5% of protein and 0-0.5% of CMC were obtained by emulsification of oil with aqueous WPI-CMC complex solutions through a two-stage process. The WPI-CMC complexes were prepared by mixing the biopolymers and water at appropriate amount and the pH was adjusted to 7.0. The WPI-CMC mixtures were stirred for at least 1h before addition of oil and emulsification. Coarse emulsions were prepared by blending 5% oil and 95% aqueous solution together using a laboratory homogenizer, Ultra Turrax T-25 (IKA Instruments, Germany) at 12,000 rpm for 1 min at room temperature. Final emulsion

samples were obtained by using an ultrasonic processor (Sonics VC 505, power 500 W, frequency 24 kHz) with a sonotrode (3 mm, approx. length 100 mm, titanium) for 5 min (30% amplitude of maximum power). Sodium azide (0.02%) was added as an anti-microbiological agent. After emulsification, the emulsions were slowly acidified to pH 5.2 by adding 0.1M HCl (50 μL at a time). The acidified emulsions were stirred for at least 1 h before analysis to allow the pH to stabilize.

3.3.4 Zeta- (ζ-) potential measurement

Measurement of ζ-potential was carried out using the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with 633 nm laser and 173^{o} detection optics at 25 ^{o}C. Each sample was diluted at a ratio of 1:250 using 5 mM phosphate buffer at pH 5.2 in order to prevent multiple scattering effects. An individual ζ-potential measurement was determined from the average of five readings taken on the same sample. All the measurements were carried out in duplicate.

3.3.5 Droplet size determination

Droplet size distributions were determined with a Coulter Multisizer (Coulter Electronics Ltd., Luton, England) at room temperature. Each sample was diluted with 5 mM phosphate buffer at pH 5.2 at a ratio of 1:1000. The volume mean diameter of the sample was used as the average droplet size. All measurements were repeated at least twice.

3.3.6 Creaming stability of the emulsion

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. The emulsion samples were stored quiescently at ambient temperature (~25 °C) for 15 days. Emulsion stability evolutions in tubes were determined by measurements of height (millimeter units) of a distinctive clear or semi-transparent bottom serum phase layer on day 5, 10 and 15 after emulsion preparation. The extent of creaming was characterized by creaming index (CI %) = $(H_S/H_T) \times 100\%$, where H_S is the height of the serum layer, and H_T is the total height of the emulsion. Each creaming index of sample was recorded in duplicate.

3.3.7 Rheological behavior measurement

Rheological behavior of fresh emulsions was measured using a Kinexus Rheometer (Malvern Instruments Ltd., Worcestershire, UK) equipped with a cone (40 mm diameter, 4^o 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 (n) and consistency coefficient (m) were calculated using the power law model. Each treatment was measured in duplicate.

3.3.8 Determination of protein surface coverage

The concentration and composition of protein adsorbed at the oil–water interface was determined according to the method described by (Ye and others 2000) with slight modification. The fresh emulsions were centrifuged at a speed of 13,000 x g for 60 min at 20 °C in a temperature-controlled centrifuge (Beckman Coulter, Inc., Fullerton, CA). The subnatants were carefully removed using a pipette and filtered through a Fisherbrand™ Qualitative P2 Grade filter paper (Fisher Scientific Inc., PA). The total protein content of subnatants was analysed by Kjeldahl method (N × 6.38). The protein surface coverage (mg/m^2) was then calculated from the mean diameter of the oil droplets determined by the Multisizer and the difference between the amount of protein used to prepare the emulsion and those measured in the subnatant after centrifugation. All the measurements were done in duplicate.

3.3.9 Statistical analysis

SPSS software (version 21, SPSS Inc., Chicago, IL) was used to analyze significant differences ($p < 0.05$) between the properties of gels by one-way analysis of variance (ANOVA). The comparisons between the mean values were evaluated by the Duncan's test.

3.4 Results and discussion

3.4.1 Formation of WPI-CMC complex on oil droplets

The influences of M_w of CMC on the ζ-potential of WPI as a function of CMC concentration were shown in Fig.1. In the absence of CMC, the net charge of the emulsion droplets was slightly negative (-2.22 mV), which demonstrated that pH 5.2 was the pH value very close to the pI of whey protein and that the net droplet charge was close to zero. The addition of CMC caused an appreciable change in the CMC concentration dependence of the net droplet charge. When CMC was added to WPI solution before emulsification, the ζ-potential of the droplets drastically became more negative even at 0.04% CMC (-26.7 to -30.2 mV). This indicated that complexes were formed between WPI and CMC and the negatively charged WPI-CMC complexes adsorbed onto the surface of the oil droplets. Increasing CMC concentration led to more negative ζ-potential, which implied that more CMC molecules interacted with WPI. When CMC concentration exceeded 0.3%, the ζ-potential appeared to be constant, suggesting that the droplet surfaces were almost saturated by WPI-CMC complexes. Similar types of electrostatic interactions between anionic polysaccharides and proteins in solutions or emulsions have been reported previously. Harnsilawat and others (2006) reported that at pH 3 and 4, the electrical charge on the emulsion droplets became more negative as the polysaccharide concentration increased, and it reached a plateau value until the surfaces of the droplets were saturated with polysaccharide. In Liu and others (2012) study, at pH 5-7, the ζ-potential of casein-coated droplets increased with the

increasing CMC concentration and it appeared to be stable as the CMC concentration exceeded 0.3%. Guzey and McClements (2007) pointed out that the charge on β-Lactoglobulin–stabilized emulsions (0.1% oil, 0.005% β-Lactoglobulin) became more negative as pectin concentration increased from 0 to 0.005%, and it became stable with further addition of pectin.

Interestingly, there was no clear difference in the ζ-potential of emulsion droplets depending on the M_w of CMC especially in emulsions containing up to 0.1% CMC. Slight differences in the ζ-potential were observed among CMC M_w at 0.3% and 0.5% CMC. The effect of CMC M_w on the formation of complexes between casein micelles and CMC was studied. Du and others (2009) reported that addition of high M_w CMC resulted in a bigger increase in ζ-potential of CMC-coated casein micelles above pH 3.7 compared to lower M_w CMC. It should be noted that the differences in the ζ-potential found in their study was mostly within 5 mV. Since the CMCs used in our study had similar charge density, it will be reasonable that there was no clear difference in their effect on the ζ-potential of the droplets. However, at 0.3 and 0.5% CMC, the ζ-potential of the droplets made from CMC 2,500k was > 5 mV larger (more negative) than that from CMC 270k.

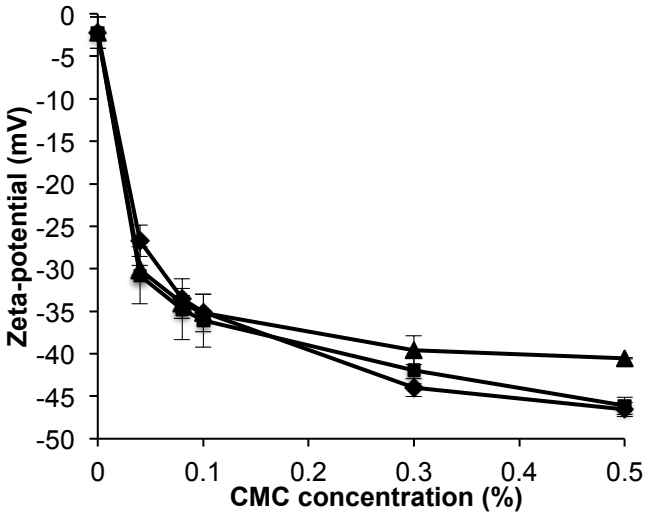


Figure 1. Dependence of electrical charge of emulsion droplets (zeta-potential) on CMC concentration and M_w. (◆) CMC 2500k; (■) CMC 750k; (▲) CMC 270k.

3.4.2 Mean particle diameter of the oil droplets

The effect of CMC concentration and M_w on the average size of the oil droplets is shown in Fig. 2. When the pH of WPI stabilized emulsion without CMC was acidified to 5.2 the mean particle diameter of the droplets significantly increased from 1.7 μm at pH 7.0 (data not shown) to 9.3 μm, indicating a high degree of droplet aggregation. This could be attributed to the fact the pH was close to the pI of the adsorbed WPI molecules, hence the electrostatic repulsion between the droplets was too small to prevent aggregation (Surh and others 2006; Li and others 2012a; Guzey and McClements 2007). The addition of CMC even at 0.04 % significantly reduced the droplet size, indicating the reduction in droplet aggregation due the overall negative charge on the surface of the droplets. This also indicated that the presence of CMC at this concentration improved the surface activity of whey protein, allowing the protein to sufficiently adsorb to the newly created oil-water interface

and prevent the formation of large droplets. This result was in agreement with other studies showing that anionic polysaccharides can reduce the aggregation of protein-stabilized emulsion droplets at near pI of the proteins (Liu and others 2012; Surh and others 2006). However, the observed large standard deviations were a clear indication that the system was unstable due to large aggregation and/or flocculation of the oil droplets (Khalloufi and others 2009). It is possible that the net charge on the droplets was at the border where stable colloid might be achieved, thus the electrostatic repulsion might be insufficient to completely prevent aggregation. In addition, CMC molecules might adsorb to the surface of more than one emulsion droplet during the emulsification, leading to bridging flocculation (Pinotti and others 1997). With increasing CMC concentration up to 0.1%, the average droplet sizes continued to decrease which corresponded to higher negative charge of the droplets. At 0.1% CMC, the average droplet sizes for CMC 2,500k, 750k and 270k were 3.7, 4.3 and 4.7 μm, respectively. At high CMC concentrations of 0.3 and 0.5%, the droplet size appeared to be stable for CMC 250k and CMC 750k, while a slight increase in droplet size was observed for CMC 2,500k. This slight increase could be due to the depletion flocculation resulted from nonadsorbed CMC molecules.

It is interesting to point out that, at ≤ 0.1% CMC, significant difference in the effect of M_w of CMC on droplet size was observed only at CMC concentrations of 0.08%. The emulsion prepared with CMC 2,500k had smaller droplet size (3.6 μm) than that prepared with CMC 270k (5.5 μm) or CMC 750k (5.5 μm). The result observed here was different with previous research which showed larger average

droplet size in emulsion prepared with higher molecular mass soy soluble polysaccharide due to the formation of thicker layer on the oil droplets. Similar phenomenon was observed by Du and others (2009) who pointed out that high M_w CMC formed thick adsorbed layer onto caseinate micelles, leading to a larger size of caseinate micelles than low M_w CMC. The negative charges of CMC were distributed along the CMC chains, yielding a conformation with many loops when adsorbed at the interface. These loops extended into the continuous phase. We speculated that higher M_w CMC with longer chain length might have more loops and greater molecular flexibility, maximizing steric stabilization, therefore, smaller droplet size. At CMC concentration of 0.3 and 0.5%, there was no change in droplet size and no difference among CMCs with different M_w. This could be due to the saturated coverage of CMC molecules on the surfaces of oil droplets and the excess CMC molecules would be present in the aqueous phase of the emulsions.

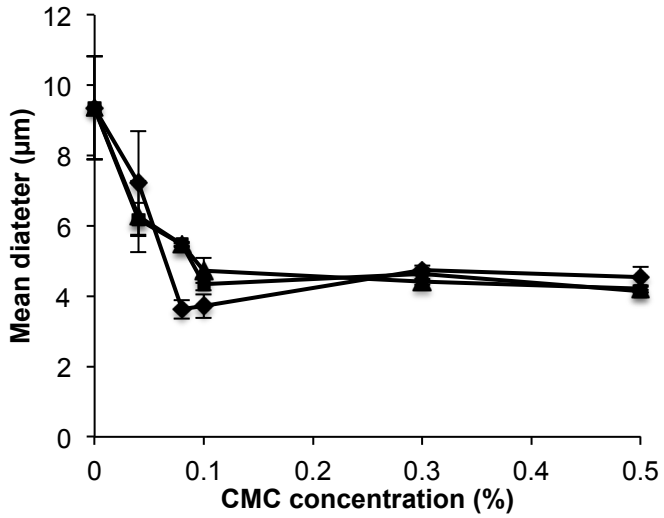


Figure 2. Dependence of mean volume diameter of emulsion droplets on CMC concentration and M_w. (◆) CMC 2500k; (■) CMC 750k; (▲) CMC 270k.

3.4.3 Emulsion stability during storage

Fig. 3 shows the effect of CMC concentration and M_w on creaming stability of WPI-stabilized emulsions during 15-day storage. Without CMC, it was not possible to prepare a stable emulsion at pH 5.2. The emulsion separated into a white cream layer at the top and a transparent serum layer at the bottom within 3 h of preparation (data not shown). This suggested that all of the droplets were aggregated and rapidly moved upwards due to gravity, which supported the ζ-potential and droplet size results. It was worthy to mention that even though the emulsion in the absent of CMC was unstable and separated rapidly, the creaming rate was much slower than that shown by other studies. Liu and others (2012) and Surh and others (2006) reported that casein-coated emulsion without polysaccharides at pH 4 separated into a cream layer and a serum layer within only a few minutes. In their studies, the emulsions were prepared by high-pressure homogenization and the average droplet size of emulsion containing casein alone was > 300 μm. In Koupantsis and Kiosseoglou (2009) study, after the emulsion prepared by high-pressure homogenization was acidified to pH 5, the average droplet diameter of the fresh emulsion containing WPI alone was about of 13 μm and the percentage of serum was more than 30% only after one-day storage. The improved emulsion stability in our study could be attributed to the smaller droplet size (9.3 μm) created by sonication compared with that in other studies. Stoke's law states that the velocity at which a droplet moves is proportional to the square of its radius, thus, by reducing the droplet size, the stability of emulsion can be increased. Ultrasound

homogenization has shown a great affinity for producing emulsions with smaller droplet size (Jafari and others 2007; Koocheki and others 2009; Delmas and others 2011). The addition of CMC had an obvious impact on creaming rate of emulsion upon acidification. Addition of CMC led to more stable emulsions. Furthermore, the effects of M_w and concentration of CMC are clearly shown.

Overall, emulsions prepared with higher M_w CMC were more stable compared to those with lower M_w CMC as shown by lower creaming rate (Fig. 3). Clearly, emulsions containing CMC with highest M_w (2,500k) were the most stable compared to those containing CMC 750k or 250k across all CMC concentrations and all storage time. Emulsions prepared with CMC 270k and 750k showed much faster creaming rate than those with CMC 2,500k within the first 5 days of preparation, and samples with CMC 270k had more rapid separation than those with CMC 750k within 10 days of preparation. These results were in accordance with previous study which showed that CMC with higher M_w resulted in a more stable acidified milk drink system than CMC with lower M_w (Du and others 2009). They explained that higher viscosity induced by the margin of the nonadsorbed high M_w CMC should contribute to the higher stability of the system. This will be discussed in rheological behavior (Section 3.4.4). Semenova (1996) suggested that increasing dextran M_w favored coacervation with soy globulin because larger size polysaccharide was more accessible for the protein. This may explain the effect of CMC 2,500k over the lower M_w CMC on forming complex with whey protein molecules, leading to reduced creaming rate and improved emulsion stability. In addition, Li and others (2012b)

pointed out that the large size of polysaccharides adsorbed onto interfaces and protruding into the continuous phase acted as a thick steric layer, which provided effective stabilization between emulsion droplets and acted in concert with electrostatic repulsive forces. This might also be an explanation for emulsion with high M_w CMC exhibited better creaming stability than that with low M_w CMC across all the CMC concentrations.

The effect of CMC concentration can be clearly observed and it also depends on the type/M_w of CMC. Addition of only 0.04% of CMC 2,500k reduced the creaming index from 55.6% to 9.2% and from 58.5% to 19.7% after 5 and 15 days of storage, respectively. Interestingly, emulsion with 0.08% CMC 2,500k was the most stable and there was no separation after 15 days of storage (Fig. 3a). This might be attributed to the fact that increasing CMC concentration up to 0.08% led to increased protein surface coverage of the droplets and thus enhanced stability (Fig. 5). This also coincided with the ζ-potential and droplet size results where increasing concentration up to 0.08% resulted in more negative charge and smaller droplet size (Figs. 1 and 2). In addition, the thickness of the interfacial layer increased due to the adsorption of CMC to the droplet surfaces, hence, both strong electrostatic repulsion and steric repulsion between the WPI/CMC-coated droplets prevent aggregation (Harnsilawat and others 2006). Further increase in CMC concentration led to less stable systems, which were demonstrated by phase separation on emulsion samples with 0.1 and 0.3% CMC. This destabilization should be related to the depletion flocculation caused by the nonadsorbed CMC molecules (Dickinson and others

1997a; Liu and others 2012). Interestingly, emulsion with 0.5% CMC 2,500k did not show any phase separation within 10 days of preparation. It could be due to the high serum viscosity of this sample and/or immobilization of dispersed oil droplets in a weak gel-like network, resulting in lighter depletion flocculation, thus, the rate and extent of phase separation may be impeded (Dickinson 2003; Long and others 2013). However, it did not contribute to the long-time stability of the emulsion system because the emulsion gradually separated after 10 days of storage. This weak network undergoes restructuring due to the Brownian motion of the oil droplets and gravity with time, causing the collapse of the gel network, eventually leading to cream separation from serum phase (Hermar and others 2001). The effect of CMC concentration on creaming stability was similar for CMC 270k and 750k. Increasing CMC concentration led to more stable emulsions and emulsion containing 0.5% CMC was the most stable one during 15-day storage (Figs. 3b and 3c). This could be due to the relatively smaller droplet size and/or higher viscosity of the serum phase. The effects of hydrocolloids on emulsification properties of protein-stabilized emulsion have been reported (Long and others 2013; Hermar and others 2001; Li and others 2012a; Singh and others 2003; Harnsilawat and others 2006). A minimum concentration of hydrocolloids is needed in order to form stable emulsion. In this case, it is clearly shown that the minimum/optimum CMC concentration needed for stable emulsion depends on the size/M_w of CMC. The CMC concentration needed for stabilizing emulsion was lower with higher M_w CMC than that with lower M_w CMC. As previously stated, it could be that high M_w CMC with longer chain would

form conformation loops extending into continuous phase, leading to more stable system through steric stabilization.

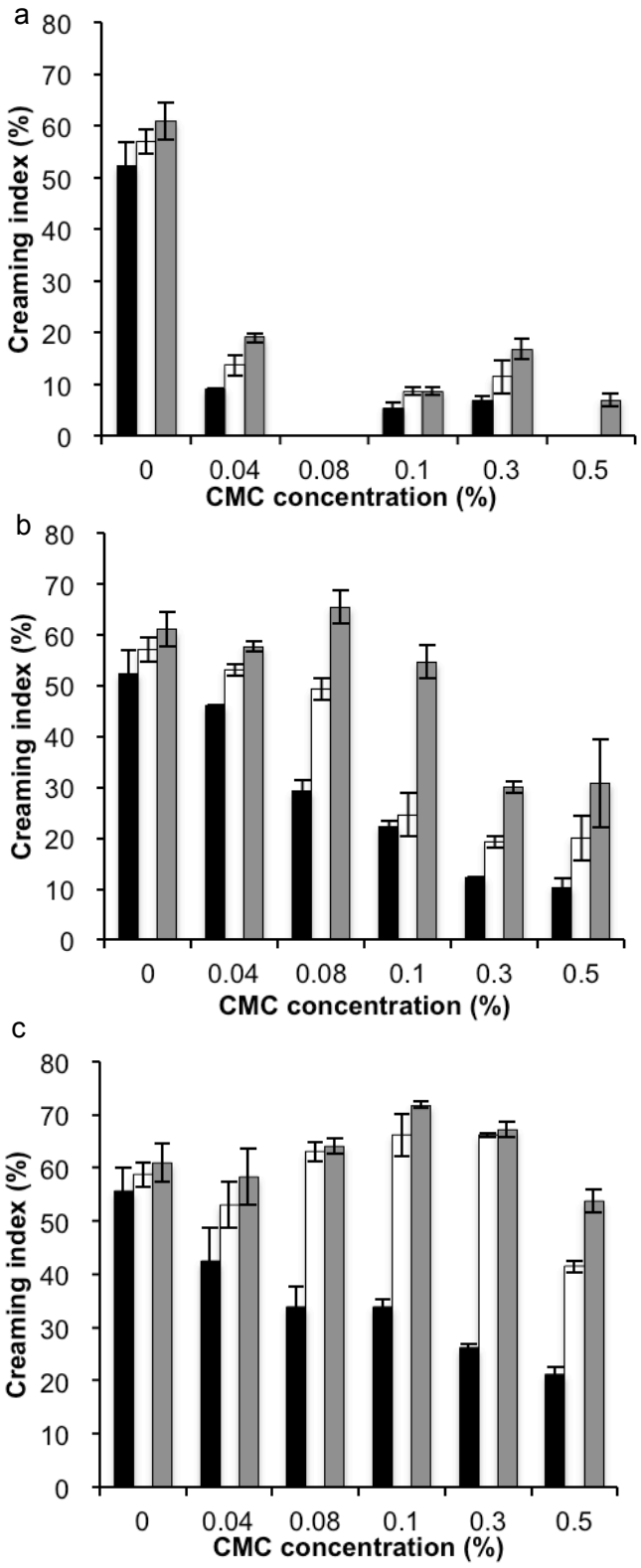


Figure 3. Creaming stability of emulsions prepared with different M_w of CMC stored quiescently for 15 days at room temperature: (a) CMC 2500k; (b) CMC 750k; (c) CMC 270k. (■) day 5; (□) day 10; (■) day 15.

3.4.4 Rheological behavior of emulsions

Flow behavior of fresh emulsions was measured immediately after the preparation of the emulsions. Plots of apparent viscosity versus shear rate for WPI/CMC-stabilized emulsions containing different concentrations and M_w of CMC are shown in Fig. 4. Power law model was applied to further describe the flow curve dispersions. The values of consistency coefficient (m) and flow behavior index (n) are listed in Table 1. Consistency coefficient is a measure of viscosity and its trend is in agreement with that of viscosity vs. shear rate presented in Fig. 4. The flow behavior index indicates the shear-thinning ($n < 1$), dilatant ($n > 1$), or Newtonian ($n = 1$) behavior.

In the absence of CMC, the emulsion exhibited a shear-thinning behavior with n value of 0.664, as the apparent viscosity decreased with the increase of shear rate, but with a turning point. This turning point might be explained by the fact that the flocs were disrupted at this shear rate, thus decreasing the effective volume fraction and lowering the viscosity with further increasing shear rate (Lorenzo and others 2008; Franco and others 1995). Similar behavior was shown in caseinate-stabilized emulsion at pH 5 that the emulsion showed strong shear-thinning behavior (Surh et al., 2006). Jourdain and others (2009) also reported that emulsion prepared with caseinate showed shear-thinning behavior at pH 6 and pH 2. With addition of CMC, the emulsions showed different flow behaviors depending on the M_w of CMC as a function of CMC concentration. As shown in Table 1, emulsions containing 0.04 and 0.08% CMC 2,500k exhibited a behavior close to Newtonian with much lower

viscosity compared with that without CMC. This result suggested that there was a high degree of droplet flocculation in emulsion prepared with WPI alone and the addition of CMC could prevent the formation of large droplets due to increased negative charge on the droplets. Increasing CMC 2,500k concentration (0.1 and 0.3%) resulted in emulsions showing shear-thinning behavior, which implied the formation of larger droplets due to more adsorption of WPI-CMC complex on the surfaces of droplets. This result was in accordance with the droplet size and creaming stability. For emulsion with 0.5% CMC 2,500k, it showed much higher viscosity, indicating that the nonadsorbed CMC increased the viscosity of serum, thus led to a relatively stable emulsion system within 10 days of preparation. Similar rheological behavior has been reported on emulsion system with high addition of polysaccharide (Long and others 2013; Sun and others 2007; Ye and others 2004).

The trend on viscosity and flow behavior of CMC 270 and 750k as a function of CMC concentration was similar Emulsions containing 0.08 to 0.3% CMC exhibited Newtonian behavior with much lower viscosity compared with those without CMC. At the highest CMC concentration (0.5%), flow behavior of emulsions with CMC 250k and 750k changed to shear-thinning with the consistency index smaller (less viscous) than that of CMC 2,500k. Consistency index of emulsions with CMC750k was larger than that with CMC 270k.

The differences in rheological behavior among emulsions with different M_w of CMC were also clearly shown (Fig. 4). Though the change in rheological behavior from shear-thinning to Newtonian (low viscosity) and back to shear-thinning (high

viscosity), the concentrations where these changes occurred were different among different M_w. The flow behavior of emulsions prepared with CMC 2,500k shifted from shear-thinning to Newtonian at 0.04%, and back to shear-thinning at 0.1%; however, the concentrations where these changes occurred for emulsions prepared with CMC 750k and 270k were at 0.08% and 0.3%. At 0.04% CMC, emulsion with CMC 2,500k showed a change in rheological behavior from shear-thinning of highly flocculated emulsion of WPI (no CMC) to Newtonian behavior with significant decreased viscosity, while those with CMC 270k and 750k still showed shear-thinning behavior. In addition, at shear rate < 1 s^{-1}, the viscosity of emulsions with CMC 2,500k and CMC 750k was lower than that without CMC, while emulsion with CMC 270k had higher viscosity than that without CMC. This might be attributed to the fact that high M_w CMC was more accessible for protein than low M_w CMC, leading to a relatively more stable system with smaller droplets, as shown by droplet size and creaming index. Even though emulsions containing 0.08% CMC showed shear-thinning behavior with similar viscosity values, they showed significant difference in creaming stability (Fig. 3). The fact suggested that the droplets were more likely to be coalesced in emulsions with CMC 270k and 750k, since coalescence leads to an increased creaming rate but has little effect on emulsion viscosity (McClements 2005). This coincided with the droplet size result, which showed emulsion prepared with CMC 2,500k had much smaller droplet size than that prepared with CMC 750k or 270k. Emulsions containing 0.3 and 0.5% CMC both exhibited shear-thinning behavior; however, the one with higher M_w

CMC had higher viscosity compared with that with lower M_w CMC. The consistency coefficient values were 0.043, 0.026 and 0.017 for emulsions with 0.5% CMC 2,500k, 750k and 270k, respectively. As shown in Fig. 1, the droplet surfaces were saturated with CMC when CMC concentration exceeded 0.3%, thus the nonadsorbed CMC molecules with high M_w would resulted in more viscous serum compared with those with low M_w, exhibiting higher viscosity of the emulsion system.

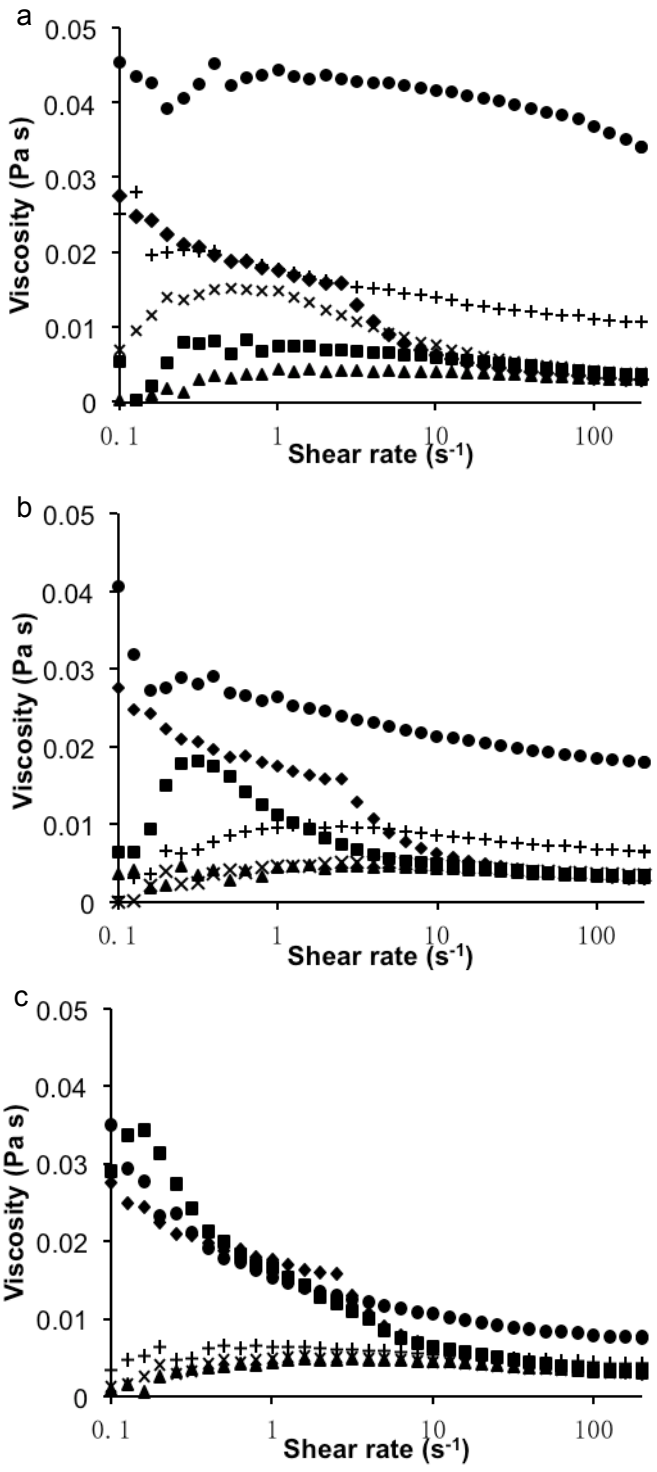


Figure 4. Apparent viscosity of fresh emulsions prepared with different CMC concentrations (◆, control; ■, 0.04%; ▲, 0.08%; ×, 0.1%; +, 0.3%; ●, 0.5%) and M_w. (a) CMC 2500k; (b) CMC 750k; (c) CMC 270k.

Table 1. Power law model parameters for emulsions with different CMC concentrations and M_w.

CMC concentration (%)		m (Pa s^n)	n	R^2
CMC 2,500k	0	0.017^{b}	0.664^{a}	0.9925
	0.04	0.005^{a}	0.968^{bc}	0.9639
	0.08	0.003^{a}	1.056^{c}	0.9741
	0.1	0.008^{a}	0.872^{b}	0.9904
	0.3	0.018^{b}	0.886^{b}	0.9981
	0.5	0.044^{c}	0.954^{bc}	0.9998
CMC 750k	0	0.017^{c}	0.664^{a}	0.9925
	0.04	0.009^{b}	0.794^{b}	0.9780
	0.08	0.004^{a}	0.999^{de}	0.9920
	0.1	0.005^{a}	0.969^{cd}	0.9832
	0.3	0.007^{ab}	1.047^{e}	0.9810
	0.5	0.025^{d}	0.919^{c}	0.9985
CMC 270k	0	0.017^{b}	0.664^{a}	0.9925
	0.04	0.012^{ab}	0.748^{a}	0.9977
	0.08	0.003^{a}	1.079^{b}	0.9517
	0.1	0.004^{a}	1.064^{b}	0.9473
	0.3	0.011^{ab}	0.861^{ab}	0.9975
	0.5	0.017^{b}	0.823^{a}	0.9966

Consistency coefficient (m), flow behavior index (n), determination coefficient (R^2). The data listed in the table were the average from two measurements. Different lower cases under the same M_w of CMC indicate significant differences ($p < 0.05$).

3.4.5 Protein surface coverage

The adsorbed protein on the surface of the oil droplets forms the viscoelastic interfacial film and determines the stability of emulsion system due to its important role in competition or cooperation with other biopolymers (Long and others 2013). Fig. 5. shows the effect of CMC concentration and M_w on the protein surface coverage of acidified WPI/CMC-coated oil droplets. It was obvious that CMC concentration showed a significant impact on protein surface coverage. The lowest protein surface coverage (0.22 mg/m^2) was observed for the emulsion without CMC addition. The protein surface coverage increased to 0.84, 0.82 and 0.49 mg/m^2 with 0.04% CMC 2,500k, 750k and 270k, respectively. It indicated that CMC improved the surface activity of whey protein, contributing to the relatively slower creaming rate and higher stability of emulsion system compared with emulsion stabilized by WPI alone. The protein surface coverage showed a continuous increase as CMC concentration increased up to 0.08 or 0.1%. This corresponded to increased ζ-potential and decreased droplet size with increasing CMC concentration up to 0.1% (Figs. 1 and 2), Formation of WPI-CMC complex resulted in a collective and cooperative adsorption of whey protein and CMC onto oil-water interface, leading to higher protein surface coverage (Li and others 2012b). Further increase in CMC concentration resulted in a slight decrease in protein surface coverage at 0.3% CMC and it either stayed constant or increased slightly at 0.5% CMC. This could be attributed to the fact that when oil droplets were saturated with CMC the presence of excessive CMC in the continuous phase would cause depletion flocculation,

resulting in the change of protein adsorption behavior.

Interestingly, protein surface coverage appeared to saturate at maximum interfacial concentration of 2.13 mg/m^2 for emulsion containing 0.08% CMC 2,500k. This highest protein surface coverage could contribute to an improvement in emulsifying functionality showing lowest creaming rate and highest emulsion stability during storage. The improvement was due to the formation of a thick and compact interfacial layer around oil droplet surface and the saturated concentration of protein, providing strong steric stabilization against aggregation/flocculation and coalescence (Dickinson and others 1997b; Li and others 2012b).

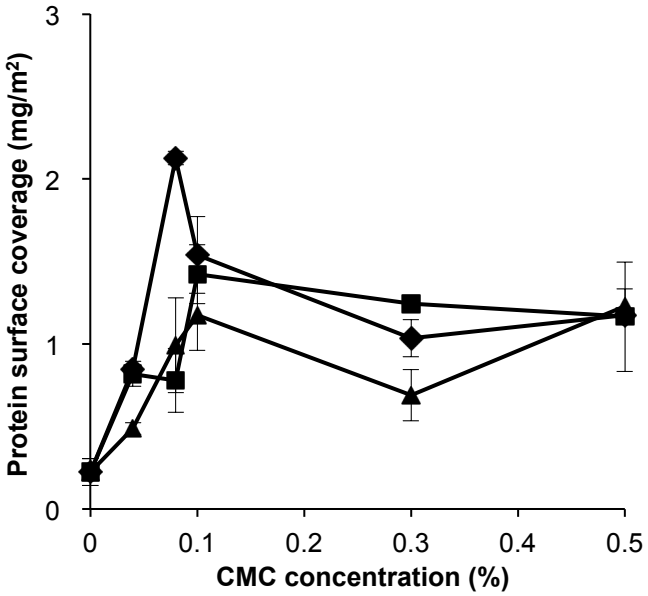


Figure 5. Protein surface coverage of emulsion droplets as function of CMC concentration. (◆) CMC 2500k; (■) CMC 750k; (▲) CMC 270k.

3.5 Conclusion

To summarize, for emulsion adjusted to pH 5.2, ζ-potential and droplet size clearly showed that, in the absent of CMC, the oil droplets formed large aggregates due to very low electrostatic repulsive force. The presence of CMC led to formation of WPI-CMC complex and resulted in improved surface activity of protein as shown by higher protein surface coverage and significantly more negative charges on the droplets. WPI-CMC stabilized emulsions showed smaller droplet size and had improved stability. Overall, increasing CMC concentration up to 0.1% enhanced the adsorption of protein at the interface and increased the negative charge of the droplets, resulting in smaller droplet size and more stable emulsions. Above 0.1% CMC, the droplet surfaces were saturated with CMC as shown by little or change in droplet size and ζ-potential, and protein surface coverage. Further addition of CMC would increase the viscosity of the continuous aqueous phase and/or form weak gel-like network. Consequently, the emulsions were more stable against creaming. However, this high viscosity or weak gel-like network did not contribute to the long-term stability of the emulsion. It could be attributed to the fact that Brownian motion of the oil droplets and gravity with time resulted in the collapse of the gel network.

Although CMC molecular weight did not show significant difference on ζ-potential or droplet size of the emulsion, it significantly influenced protein adsorption as well as the flow behavior and the stability of the emulsions. Overall, higher M_w CMC contributed to more stable emulsions compared to lower M_w CMC

according to the creaming index. This was likely due to combined effects of increased protein surface coverage on the droplets and viscosity of the emulsions. Viscosity effect strongly dominated at high CMC concentration with emulsions containing high M_w being the most stable. Therefore, the stability of acidified WPI/CMC-stabilized emulsion was related to CMC concentration, CMC molecular weight, droplet size, viscosity of the system and protein surface coverage of the droplets. Complexation between WPI and CMC even at pH > pI provided improved the surface properties of the protein and enhanced the electrostatic as well as steric repulsion of the adsorbed layers, contributing to the stability of acidified WPI/CMC-stabilized emulsions.

CHAPTER 4

ACID-INDUCED GELATION OF HEATED SOLUBLE WHEY PROTEIN ISOLATE-CMC COMPLEX

Manuscript to be Submitted for Publication

4.1 Abstract

Acid-induced gelation of heated soluble whey protein isolate (WPI) and carboxymethylcellulose (CMC) complex was investigated. Heated soluble WPI-CMC complexes were prepared by mixing the biopolymers at pH 7 and heated at 85 ^{o}C for 30min. Gels were formed by the addition of glucono-δ-lactone (GDL) and compared to those formed from WPI polymer (protein heated alone) and added CMC. All gels contained 5% (w/w) protein and 0-0.125% (w/w) CMC (M_w = 270k, 680k, and 750kDa). Results showed that CMC molecular weight and biopolymer ratio were the major factors affecting gel properties. For 270k and 750k CMC, gels from heated WPI-CMC complex showed improved gel hardness and, at certain CMC concentration, improved water holding capacity. Confocal laser scanning microscopy (CLSM) results revealed that gel structure largely depended on CMC concentration. Overall, gels from heated WPI-CMC complex showed smoother structure and less porosity, indicating less phase separation. Furthermore, significantly higher gel hardness and water holding capacity were observed across all CMC concentrations when heated WPI-CMC complexes (CMC 750k) were

formed at initial protein concentration of 9%. CLSM image showed bi-continuous microstructure even at 0.1% CMC where phase inversion was observed for gel from 6%WPI-CMC complex.

4.2 Introduction

Whey protein isolate (WPI) is widely used in many food products due to its unique functional properties and high nutritional values. Among its many functional properties, gelation of WPI has been studied extensively (Alting and others 2000; Britten and Giroux 2001; Li and others 2006; Cavallieri and da Cunha 2008; Clark and others 1981; Foegeding and others 1998; Vardhanabhuti and others 2001; Çakır and others 2012). Cold-set gelation is a two-step gelation process conducted at ambient temperature. In the first step, protein polymers are obtained by heating the protein solution at below critical gelation concentration, pH above or below the isoelectric point, and low ionic strength. In the second step, acid or salt is added to the protein polymer solution to induce gelation by reducing the electrostatic repulsion between the protein aggregates (Bryant and McClements 2000b; Ju and Kilara 1998; de Jong and others 2009; Kuhn and others 2010; Hongsprabhas and Barbut 1997b; de Faria and others 2013).

Polysaccharides are added to alter the functional properties of food proteins. Attractive interactions between protein and polysaccharide can lead to soluble and/or insoluble complexation, cosolubility or segregation (Schmitt and others 1998; Rodríguez Patino and Pilosof 2011). Functional properties of protein are generally

improved by complexation with other polysaccharides. Their interactions are mainly affected by pH, ionic strength, protein to polysaccharide ratio, and biopolymer concentrations. Some other factors such as biopolymer characteristics (charge density, molecular weight, et al.), temperature and pressure also influence the complex formation (Schmitt and Turgeon 2011). Protein-polysaccharide electrostatic complex generally occurs when the biopolymers are mixed at pH values below the pI of the proteins and at low ionic strength (Ye 2008; Turgeon and others 2007). However, at pH values above the pI of proteins, e.g. at neutral pH, electrostatic interactions between negatively charged polysaccharides and positively charged subunits of proteins can still occur (Doublier and others 2000; Vardhanabhuti and others 2009; Girard and others 2002a). Heating mixed biopolymers at this condition can result in heated soluble complex having different size and shape compared to heated protein aggregates without polysaccharides. de la Fuente and others (2004) reported lower molecular weight of whey protein/κ-carrageenan aggregates formed in the early stages of heating at pH 7 than the aggregates formed without κ-carrageenan. At near neutral pH, heating mixtures of whey protein with low methoxyl pectin resulted in a complex with smaller molecular weight than heated whey protein alone (Beaulieu and others 2005). When formed at appropriate conditions, heated soluble protein-polysaccharide complex could also offer better functional properties. It has been reported that dextran sulfate improved thermal stability of β-lactoglobulin by altering its aggregation and the complexation between the two by heating at near neutral pH could form solutions with lower turbidity or

gels (Vardhanabhuti and others 2009). Zhang and others (2014) recently demonstrated that acid-induced gels formed from heated soluble whey protein/pectin complex at pH 7 (e.g., heated together) had improved gel strength and water holding capacity compared to those formed from whey protein polymer with added pectin (e.g., heated separately). Enhanced gel properties were supported by finer gel microstructure with less porosity and smoother network.

Carboxymethylcellulose (CMC),derivative of cellulose, is a common water-soluble anionic polysaccharide used in the food industry. CMC has the ability to impart viscosity to aqueous solutions. The viscosity of CMC is determined largely through controlling cellulose chain length or degree of polymerization (DP). A maximum degree of substitution (DS) of 1.5 is permitted, but for food applications, DS is in the range 0.6-0.95 (Coffey and others 2006; Murray 2000). Both DP and DS determine the molecular weight of CMC. It is believed that molecular weight of polysaccharides would have effects on their interactions with protein and how they affect functional properties of protein. Du and others (2009) reported that molecular weight of CMC influenced the interaction between casein micelles and CMC. Acidified skim milk drinks with high molecular weight CMC had better long-term stability compared to those with low molecular weight CMC. The majority of the studies have focused on protein-CMC complex/coacervates forming at pH below or near protein pI. Protein-CMC complex formation at a pH near protein pI promotes the stabilization of protein in acidified protein beverages and yogurt drinks (Du and others 2007; Koupantsis and Kiosseoglou 2009) and had greater emulsifier

properties than protein alone (Girard and others 2002b). Whey protein complexation with CMC showed maximum thermostability at pH 4 and gelation properties were improved upon heating when compared with non-complexed whey protein (Capitani and others 2007). To the best of our knowledge, no study has investigated the functional properties of heated soluble whey protein-CMC complex forming at neutral pH.

The objective of this study was to investigate the acid-induced gelation of heated soluble WPI-CMC complex. The effects of CMC molecular weight (M_w) and concentration were studied. Physical properties of the heated soluble complexes (e.g., particle size and zeta-potential) and gels (e.g., water holding capacity and gel hardness) were measured. The microstructure of the gels was determined by confocal laser scanning microscopy (CLSM).

4.3 Materials and methods

4.3.1 Materials

Whey protein isolate (WPI) Bipro^{TM} was kindly provided by Davisco Foods International Inc. (Le Sueur, MN). According to the manufacturer, WPI contained 97.9% protein on dry basis and 1.8% ashes of the dry mass. CMC with molecular weight (M_w) of 270k, 680k, and 750kDa were kindly provided by CP Kelco Inc. (Lille Skensved, Denmark). D-(+)-Gluconic acid δ-lactone (Sigma^{TM} GDL) and Rhodamine B were purchased from Sigma (St. Louis, MO). All ingredients were

used without further purification and without correction for their moisture content. Deionized (DI) water (> 18.2 MΩ) was used in all cases.

4.3.2 Preparation of stock solutions

WPI stock solution (10%, w/w) was prepared by slowly dissolving protein powder into DI water and kept stirring at the room temperature for at least 2 h. CMC stock solution (0.9%, w/w) was prepared by slow addition of CMC powder into DI water heated at 85 ^{o}C for 1 h under continuous stirring. The two stock solutions were stored at 4 ^{o}C in the refrigerator overnight for complete hydration. WPI and CMC stock solutions were warmed to ambient temperature before use.

4.3.3 Gel preparation

WPI-CMC complex. Stock solutions of WPI and CMC were mixed at appropriate amount and pH was adjusted to 7.0. The final concentration of protein in the mixed solution was at 6%, with CMC concentration ranged from 0 to 0.15%. The mixture was kept stirring at room temperature for 2 h for completely mixing before heated in the water bath at 85 ^{o}C for 30 min and cooled using running tap water. DI water was added such that the final protein concentration was at 5% and CMC concentrations ranged from 0 to 0.125%.

Polymer-CMC. Whey protein polymer solution was prepared by heating 6% WPI solutions at pH 7.0 at 85 ^{o}C for 30 min. After polymer solution was cooled using running tap water, CMC stock solution was added at the appropriate amount. The pH of the samples were adjusted to 7.0 and DI water was added so that the final protein

concentration was 5% and CMC concentration ranged from 0 to 0.125%. Samples were gently stirring at room temperature for 2 h for completely mixing.

All samples were left in the refrigerator for 18 h for complete interaction between protein and CMC. GDL was added to both WPI-CMC complex and polymer-CMC mixed solutions with different WPI/GDL ratio to reach a final pH of 4.7 ± 0.1 after 24 h incubation. All the measurements were carried out within 20 to 24 h after addition of GDL.

4.3.4 Particle size and zeta- (ζ-) potential

Measurements of particle size and ζ-potential were carried out using the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with 633 nm laser and 173^{o} detection optics. Samples were diluted with DI water to protein concentration of 0.3%. Z-average diameter of the particles was used as the effective diameter. An individual ζ-potential measurement was determined from the average of three readings taken on the same sample. Each measurement was carried out twice.

4.3.5 Gel hardness

WPI-CMC complex and polymer-CMC (36 g) formed 30-mm-thick gels. Large deformation tests were performed using a texture analyser (TA-Hdi, Texture Technologies Corp, Scarsdale, NY) with a 5-kg load cell and 13-mm-diameter cylindrical plunger. Before analysis, samples were equilibrated at ambient temperature for 2 h. The penetration distance was fixed to 10% of the original gel

thickness with a deformation rate of 10 mm/s. The force required to maintain the 10% strain was recorded for 300 s. Gel hardness was expressed as the initial force (g) at the maximum peak of the force-time curve (Bourne and others 1978). All the treatments were run in triplicate.

4.3.6 Water holding capacity

Gel samples (1.5 g) were formed in the microcentrifuge tubes for measurement of water holding capacity. Loss of water was determined after centrifugation using a microcentrifuge (Eppendorf Minispin® Centrifuge) at 10 000 × g for 10 min. Water holding capacity (WHC, %) is expressed as percentage of water retained after centrifugation. Each sample was analyzed in triplicate.

4.3.7 Confocal laser scanning microscopy (CLSM)

Samples for CLSM imaging were mixed with Rhodamine B solution (20 μL of a 0.2 % solution/g of sample) before acidification. After GDL addition, 70 μL of dyed solution was pipetted onto micro slides with 0.17 mm coverslips. Samples were left to gel at 4 ^{o}C and CLSM images were recorded at room temperature using a Zeiss LSM 510 META confocal laser scanning microscope (Cal Zeiss, Jena, Germany) with 63 × water immersion objective. The excitation wavelength was 543nm. Digital image files were acquired in 1024 pixels × 1024 pixels. Z-stacks of xy-scans were recorded between 6 and 50 μm penetrations, with an interval of 3 μm. All the reported images in this paper were recorded at a penetration depth of 18 μm.

4.3.8 Statistical analysis

SPSS software (version 21, SPSS Inc., Chicago, IL) was used to analyze significant differences ($p < 0.05$) between the properties of gels by one-way analysis of variance (ANOVA). The comparisons between the mean values were evaluated by the Duncan's multiple range test.

4.4 Results and discussion

4.4.1 Particle size and zeta- (ζ-) potential of mixed solutions

In order to confirm the formation of heated soluble complex at neutral pH, particle size of WPI-CMC complex and polymer-CMC solutions was measured (Table 2). When WPI and CMC were heated together, particle size distribution of WPI-CMC complex showed one single peak with the size between that of native WPI and CMC. The peaks of native WPI and CMC also disappeared, suggesting the formation of heated soluble complex between WPI and CMC. Similarly, particle size distribution of heated soluble WPI and pectin complex (heated together at near neutral pH) also showed a single peak, while the native WPI and pectin peaks disappeared (Zhang and others 2014). For polymer-CMC, particle size distribution also showed a single peak after the biopolymers were mixed, indicating that WPI polymer and CMC also associated. It has been reported that at pH > 6, chain segment model of binding occurred between positively charged segments in BSA molecules and anionic polysaccharides forming complex when heated them together, reflecting by the BSA peak overlapped the peak of sodium alginate or pectin

conduced by gel filtration (Cai and Arntfield 1997).

Results shown in Table 1 indicate that increasing CMC concentration led to the formation of slightly larger heated complex compared to WPI polymers without CMC. Zhang and others (2014) found that at pH 7, heating WPI together with pectin led to the formation of aggregates with larger size and/or with different shape compared with polymer/pectin. There was no difference in particle size between WPI-CMC complex and polymer-CMC at the same CMC molecular weight and concentration. In addition, CMC molecular weight did not seem to have any effect on particle size.

ζ-potential measurement was used to investigate the surface charge properties of the particles (Table 3). Addition of CMC resulted in an increase in net negative charges on both WPI-CMC complex and polymer-CMC particles. An increase in the repulsive force between protein molecules led to micro phase separation, which could explain the observed larger particle size of the aggregates at higher CMC concentration. There appears to be a trend of increased zeta-potential with increasing CMC concentration for WPI-CMC complex and polymer-CMC. At 0.1% CMC, ζ-potential of WPI-CMC complex and polymer-CMC was significantly different from WPI polymer without CMC addition. At pH 7, though WPI and WPI polymer had net negative charge, portions of the protein molecules could be positively charged so that binding occurs between CMC and a positively charged site segment on the WPI (Dickinson 1998; Cai and Arntfield 1997). When compared WPI-CMC complex to polymer-CMC at the same CMC concentration, no difference in

ζ-potential was observed. Due to the same charge density of these three CMC, no clear effect of CMC molecular weight on the ζ-potential was found.

Table 2. Z-average particle diameter (nm) of WPI-CMC complex and polymer-CMC solutions.

	CMC concentration (%)			
	0	0.01	0.05	0.1
polymer-CMC 270k	40.2±2.2^{ab}	40.6±0.0^{abA}	43.3±0.4^{bcdA}	45.9±0.5^{cdAB}
WPI-CMC complex		39.6±1.8^{aA}	43.1±1.8^{bcA}	46.4±0.5^{dAB}
polymer-CMC 680k	40.2±2.2^{a}	39.4±1.5^{aA}	43.2±0.8^{aA}	43.8±1.0^{aA}
WPI-CMC complex		39.9±2.0^{aA}	44.1±1.9^{aA}	49.9±3.3^{bB}
polymer-CMC 750k	40.2±2.2^{a}	41.0±3.7^{aA}	44.8±3.2^{abA}	49.5±4.3^{bAB}
WPI-CMC complex		41.9±3.0^{aA}	43.6±0.9^{abA}	49.7±1.0^{bAB}

Average of duplicate measurements, ± means standard deviation.
Different lower cases under the same M_w of CMC and different upper cases at the same CMC concentration indicate significant differences ($p < 0.05$).

Table 3. Zeta-potential of WPI-CMC complex and polymer-CMC solutions.

	CMC concentration (%)			
	0	0.01	0.05	0.1
polymer-CMC 270k	-28.0±1.7^{b}	-28.1±0.5^{bA}	-29.9±1.7^{abA}	-31.0±1.2^{abB}
WPI-CMC complex		-28.5±1.8^{abA}	-30.2±1.0^{abA}	-31.9±1.6^{aAB}
polymer-CMC 680k	-28.0±1.7^{c}	-28.0±0.5^{cA}	-30.1±0.9^{cA}	-33.4±1.7^{abAB}
WPI-CMC complex		-28.3±1.2^{cA}	-31.0±1.6^{bcA}	-35.0±0.9^{aA}
polymer-CMC 750k	-28.0±1.7^{c}	-29.2±2.0^{cA}	-31.3±2.1^{bcA}	-33.1±1.8^{abAB}
WPI-CMC complex		-29.5±0.0^{bcA}	-33.2±0.3^{abA}	-35.2±1.0^{aA}

Average of duplicate measurements, ± means standard deviation.
Different lower cases under the same M_w of CMC and different upper cases at the same CMC concentration indicate significant differences ($p < 0.05$).

4.4.2 Gel hardness

All samples formed opaque gels after acidification to pH 4.7. Mechanical property of the gels was characterized by determining gel hardness. Fig. 6 shows the gel hardness as a function of CMC concentration and different M_w on WPI-CMC complex and polymer-CMC. The observed differences in gel hardness appeared to be from combined effects of CMC molecular weight and concentration.

All acid-induced gels containing CMC 270k were self-supporting. Increasing CMC concentration resulted in an increase in gel hardness across all CMC concentrations for both WPI-CMC complex and polymer-CMC gels. When compared to polymer-CMC gels at the same CMC concentration, WPI-CMC complex gels were stronger across all CMC concentrations. For CMC 680k, increasing CMC concentration led to an increase in gel hardness until it reached a maximum and then decreased at higher CMC concentrations. This could be explained by the fact that micro-phase separation forces the protein and CMC into local areas of increased concentration, leading to stronger gels. This will be further discussed in Section 3.4. Gels of polymer-CMC at CMC concentration of 0.125% was no longer self-supporting, gel hardness could not be measured accurately. No significant improvement of gel hardness was obtained for WPI-CMC complex compared with that for polymer-CMC at CMC concentrations lower than 0.1%. However, improvement in gel hardness in WPI-CMC complex can be observed at ≥ 0.1% CMC where gels from WPI-CMC complex were stronger (0.1% CMC) or self-supporting (0.125% CMC). Similar trend in the effect of CMC concentration on

gel hardness was found with CMC 750k. However, WPI-CMC complex gels showed higher gel hardness than polymer-CMC gels across all CMC concentrations. The maximum gel hardness was achieved at 0.05% CMC for both systems, and further increase in CMC concentration resulted in weaker gels. Gels containing 0.125% CMC were very weak such that they could not be measured. Our results are in agreement with those found in studies investigating the effect of polysaccharides on acid-induced gelation of WPI. de Jong and van de Velde (2007) investigated the mechanical properties of WPI/CMC (M_w = 730k) acid-induced gels and reported that large deformation characteristics of gel samples with CMC concentration higher than 0.15% were too weak to measure. This was attributed to the competition between the gel formation of the protein and the phase separation process between protein and polysaccharide. With increasing CMC concentration, net negative charge increases and phase separation process between protein and CMC overwhelms the gel formation of the protein, resulting in phase inversion and non-self-supporting gels. Similar phenomenon could describe our observation that WPI/CMC acid-induced gels were not self-supporting at higher CMC concentrations for CMC 680k and CMC 750k.

It is clearly illustrated that both CMC concentration and M_w influenced gel hardness. Furthermore, gels prepared with different M_w of CMC showed different trends in gel hardness as the function of CMC concentration. This difference was due to the microstructure of the gels; therefore, the results of gel hardness and water holding capacity will be further discussed under the microstructure section.

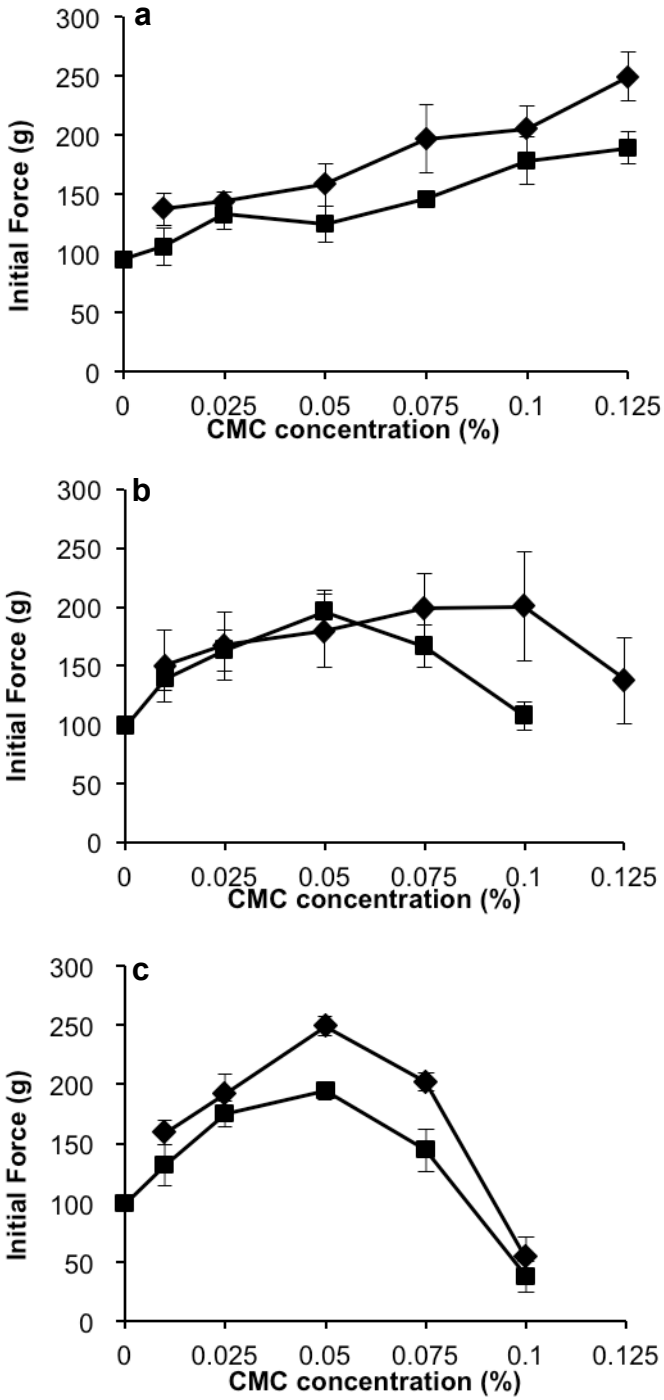


Figure 6. Effects of M_w and CMC concentration on gel hardness from WPI-CMC complex (◆) and polymer-CMC (■). (a) CMC 270k; (b) CMC 680k; (c) CMC 750k.

4.4.3 Water holding capacity

Water holding capacity is known as one of the most important characteristics of gels. It indicates the water binding ability of proteins and is generally used to objectively evaluate the quality of yogurt and other gel-based food products. Fig. 7 shows the effect of CMC concentration and molecular weight on the WHC values. Because the gels were not self-supporting at 0.125% CMC 680k and CMC 750k, the WHC values could not be measured. In the presence of 0.01 and 0.025% CMC, all acid-induced gels had > 85% WHC and there was no significant difference between WPI-CMC complex and polymer-CMC. At 0.05% CMC, WHC started to decrease to a lesser or larger degree depending on the samples. Similarly, Zhang and others (2014) reported that for gels prepared at pH 7, addition of pectin at lower than 0.15% did not have significant effect on WHC while higher pectin concentrations led to a significant decrease in WHC. It was proposed that CMC at higher concentrations enhanced the interaction between protein and polysaccharide, leading to less hydrophilic sites remained on the protein for water binding (Zhang and others 2014). It should be noted that the significant difference in WHC between WPI-CMC complex and polymer-CMC was observed with CMC 750k at 0.05% CMC. At 0.075% CMC, the WHC values of WPI-CMC complex gels containing CMC 270k, CMC 680k and CMC 750k were 51.7%, 36.6% and 28.1%, respectively and the WHC values for polymer-CMC were 41.3%, 33.3% and 26.0%, respectively. These indicated that the M_w of CMC also had an effect on gel water holding capacity, at high CMC concentrations with higher molecular weight resulting in lower WHC

values. It might be due to the fact that high M_w CMC results in more phase separation, as revealed by the microstructures.

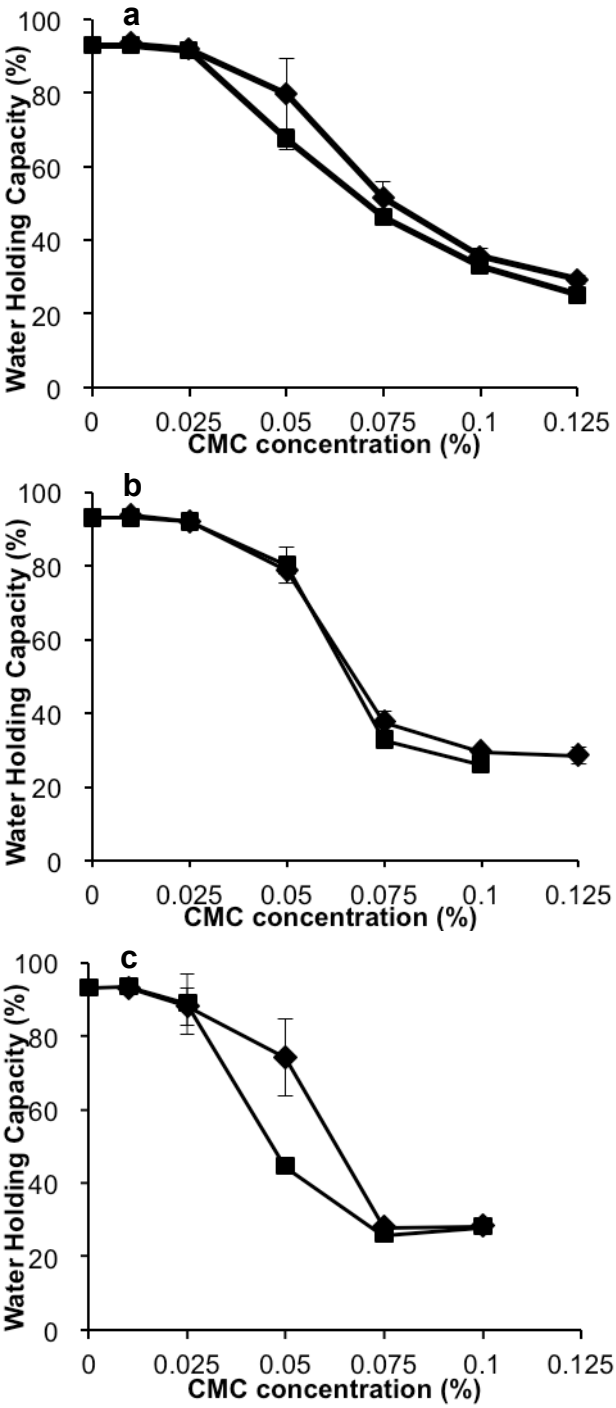


Figure 7. Effects of M_w and CMC concentration on water holding capacity of gels from WPI-CMC complex (◆) and polymer-CMC (■). (a) CMC 270k; (b) CMC 680k; (c) CMC 750k.

4.4.4 Microstructure of gels

Acid-induced gels with CMC concentrations of 0.01, 0.05 and 0.1% were selected as representative samples for CLSM analysis based on water holding capacity and gel hardness results. The CLSM images in Fig. 8 show the effects of CMC molecular weight and concentration on the network structure of WPI acid gels. At 0.01% CMC, all samples were homogeneous and no phase separation was observed through CLSM. All gels at this CMC concentration showed similar microstructure to gel made from whey protein polymer without CMC (data not shown). Clear bi-continuous networks of gels, both the protein phase and the serum phase were continuous, were observed in gels containing 0.05% CMC 250k with the WPI-CMC complex gel showing more interconnected, less phase-separated, and less porosity network. At 0.1% CMC, the protein strands were still interconnected but higher phase separation especially in polymer-CMC gels could be observed. These results were in agreement with the gel hardness and water holding capacity results. Gels containing 0.05% CMC 650k and 750k showed similar structure to those containing CMC 250k with bi-continuous and phase-separated network. At 0.1% CMC, higher phase separation can be observed in gels containing 650k. For gels containing CMC 750k, phase inversion was observed at 0.1% CMC in both polymer-CMC and WPI-CMC complex gels. de Jong and van de Velde (2007) pointed out that an increase in the concentration of intermediate charged polysaccharides resulted in coarsening of the protein network and this coarseness was enhanced with high molecular weight polysaccharide. What we found was that

at 0.1% CMC, the extent of micro-phase separation was clearly different with regard to M_w of CMC. The larger the molecular weight, the more extensive phase separation. For CMC 270k, the gel had a good network while for CMC 750k, the protein network was discontinuous and the number of effective strands was nearly zero. The remarkable difference in gel microstructures demonstrated that M_w of CMC had influence on the formation of acid-induced gels, leading to different microstructures. Monteiro and others (2005) also found the size of the polysaccharide-rich areas and the degree of connection between them increased with increasing LBG concentration or molecular weight, which meant that higher LBG molecular weight and concentration led to a more phase-separated system.

When compared the microstructure of WPI-CMC complex with polymer-CMC, gels from heated WPI-CMC complex showed smoother structure and less porosity, indicating less phase separation than polymer-CMC gels. Zhang and others (2014) concluded that heating whey protein and pectin together at pH 7 resulted in gels with less porosity microstructures and smoother networks compared with those from polymer/pectin. Heating WPI together with CMC likely enhanced biopolymer interactions, leading to improved gel strength, water holding capacity and other gel physical properties.

The differences in gel microstructures were in agreement with gel hardness as well as water hold capacity mentioned above. Addition of CMC at low concentration had little effect on WHC while high CMC addition had adverse effect on WHC due to thermodynamic incompatibility of protein and CMC. For CMC 270k, with the

increasing of CMC concentration, the phase separation became more pronounced and micro-phase separation forced the protein and polysaccharide into local areas of increased protein concentration, leading to stronger gels (de Jong and van de Velde 2007). In gels with 0.1% CMC 750k, when the phase inversion appeared, protein networks could not form, resulting in very soft gels in which water could not be entrapped.

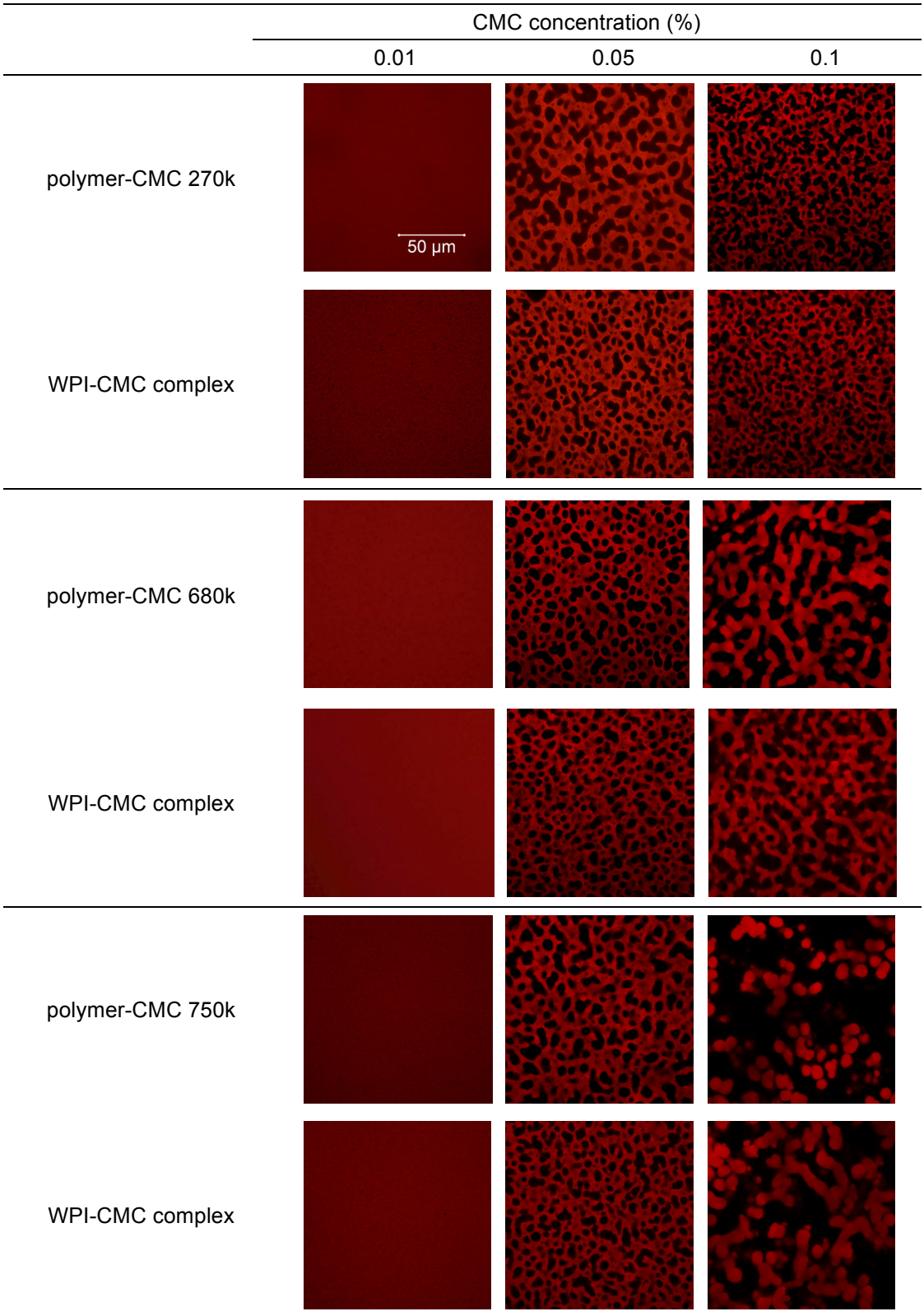


Figure 8. CLSM images of gels formed from CMC with different M_w and at different CMC concentrations.

4.4.5 Gel properties of WPI-CMC complex heated at higher protein concentration

In protein and polysaccharide interactions, biopolymer ratio and concentration both play an important role in the degree of interactions and the resulting functional properties. We have shown the effect of polysaccharide molecular weight and concentration on acid-induced gels formed from heated soluble complex. Based on the above results, we further investigated the gel properties of WPI-CMC complex heated at higher protein concentration (9%) using CMC 750k.

All acid-induced gels formed from heated 9%WPI-CMC complex were self-supporting without any syneresis. Interestingly, increasing CMC concentration resulted in a significant increase in gel hardness up to about 0.05% CMC and gel hardness then appeared to be stable (Fig. 9a). This trend was very different from that of heated 6% solutions where phase-separation led to weak gel structure. Gels formed from solutions heated at 9% protein had higher gel hardness than those formed from heated 6% protein solutions across all CMC concentrations (Fig. 9a). Significant difference can be observed at 0.1 and 0.125% CMC where gels from heated 6% solutions were weak (0.1% CMC) or too weak to be measured (0.125% CMC).

Acid-induced gels formed from heated 9% and 6% solutions also showed different trend in the effect of CMC concentration on gel water holding capacity (Fig. 9b). As discussed above, WHC of gels from heated 6% solutions decreased significantly at $\geq$ 0.05% CMC. All gels formed from heated 9% solutions showed high WHC even at the highest CMC concentration (0.125%). Microstructures of gels

formed from 9%WPI-CMC complex were also analyzed by confocal laser scanning microscopy. CLSM images are shown in Fig. 10. Gels without CMC and with 0.01% CMC were homogenous, and no differences could be observed visually. The microstructures were significantly different between 6%WPI-CMC and 9%WPI-CMC complex at 0.05 and 0.1% CMC concentrations. At 0.05% CMC, both gels had an isotropic bi-continuous microstructure where the serum phase formed continuous channels through the protein phase; however, the protein strands in 9%WPI-CMC complex gel were larger and more interconnected with much smaller pores. At 0.1% CMC, gel from 6%WPI-CMC complex showed phase inversion, while 9%WPI-CMC complex gel still showed protein as continuous phase with substantial degree of interconnected networks.

The effect of protein concentration on properties of acid-induced WPI gels without polysaccharides was investigated (de Jong and van de Velde 2007). They determined the gel hardness by large deformation and the results revealed that increasing initial protein concentration when heated resulted in an increased local concentration of protein, leading to higher firmness of the gels. Braga and others (2006) reported that the mechanical properties and WHC of acid-induced caseinate gels were improved as the initial caseinate concentration increased from 2% to 6%. When applied whey protein polymers into yogurt, Britten and Giroux (2001) found that increasing protein content reduced expressible serum which implied the improvement of water holding capacity of yogurt. The improved physical properties of the gels formed from heated 9% complex solutions may be attributed to the higher

number of thiol groups formed during heating and after acidification. Gels heated at 9% protein had more thiol groups than those heat at 6% protein, resulting in the formation of more additional disulfide bonds during gelation. Alting and others (2004) concluded that the formation of disulfide bonds had great contribution to the mechanical properties of acid-induced WPI gels. It has been reported that at higher protein concentration the acid-induced egg white gels became tougher due to more additional disulfide bonds formed after acidification (Weijers and others 2006). The hardness of acid-induced WPI gels was determined mainly by the number and accessibility of thiol groups rather than size of the aggregates or other structural features (Alting and others 2003a).

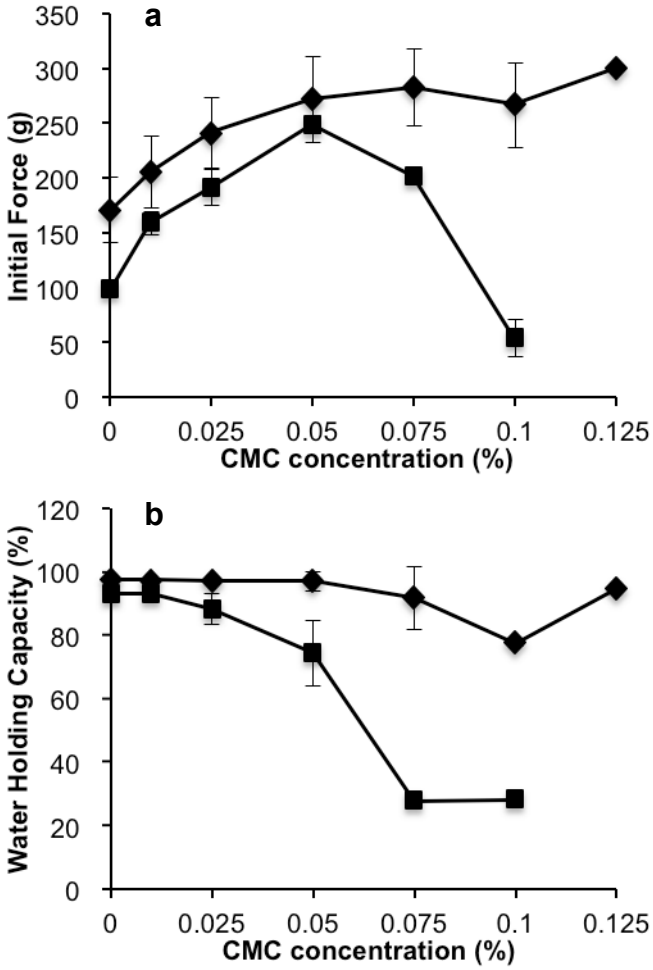


Figure 9. Physical properties of gels from 9%WPI-CMC complex (◆) and 6%WPI-CMC complex (■) (CMC 750k). (a) gel hardness; (b) water holding capacity.

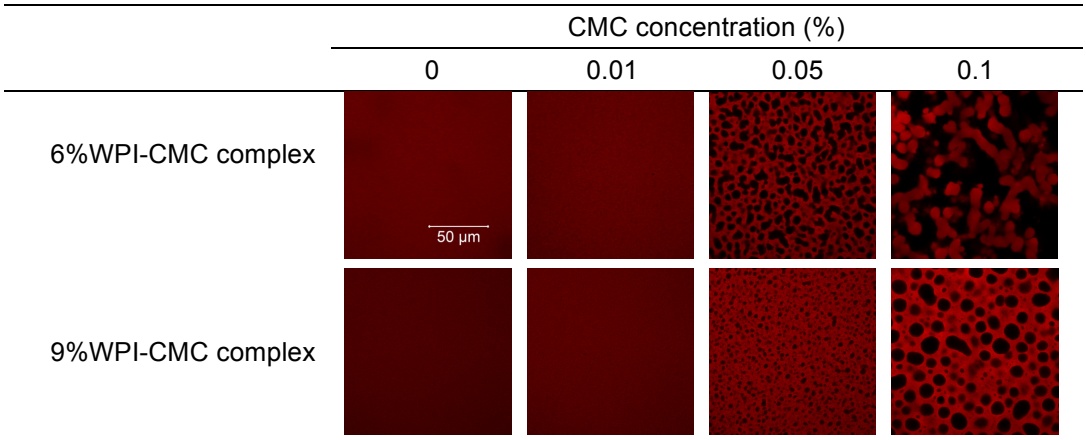


Figure 10. CLSM images of gels formed from 6%WPI-CMC complex and 9%WPI-CMC complex (CMC 750k) at different CMC concentrations.

4.5 Conclusion

In general, acid-induced gels formed from WPI-CMC complex had had higher gel hardness compared to those formed from polymer-CMC. The significant difference in WHC between WPI-CMC complex and polymer-CMC was only observed with CMC 750k at 0.05% CMC; however, microstructure analysis showed less phase-separated structure in gels from WPI-CMC complex. The CMC molecular weight and biopolymer ratio were the major factors affecting the properties of acid-induced WPI-based gels. The water holding capacity and gel hardness were CMC concentration dependent. Nevertheless, with different molecular weight of CMC, gel hardness showed significant different trends as a function of CMC concentration. It meant that molecular weight of CMC played a major role other than CMC concentration in gel formation process, leading to the disparities among the microstructure of gels.

When heated WPI-CMC complex at 9% protein, higher WHC and gel hardness values were shown compared to those from heated at 6% protein. This was due to the formation of more additional disulfide bonds during acidification and gelation process. Covalent bonds were the main determinants of hardness and other physical properties of the acid-induced WPI gels. In conclusion, this study demonstrated that gelation properties of whey protein/CMC mixed systems can be improved by heated WPI-CMC soluble complex. Heating whey protein and CMC together to form the soluble complex promoted the interactions between the two biopolymers, which contributed to the reduced the micro-phase separation with smoother and less porosity of gel structures, thus improving gel hardness and water holding capacity.

CHAPTER 5

CONCLUSIONS

This research demonstrated that formation of both unheated and heated WPI-CMC soluble complexes at near neutral pH (pH > pI) can improve emulsion stabilization and acid-induced gelation properties of the protein. Molecular weight and concentration of CMC significantly affect the properties of the complexes. High M_w CMC at optimum concentration resulted in the improvement of emulsion stability and gel properties.

5.1 Emulsification of soluble WPI-CMC complex

Modification of protein to provide good emulsification and emulsion stabilization near its pI is needed in the food and beverage industry. We have shown that complexation of WPI and CMC can lead to stable emulsion at pH 5.2. In the absence of CMC, the oil droplets formed large flocs due to very low electrostatic repulsive force. The presence of CMC led to the formation of WPI-CMC complex and resulted in improved surface activity of protein as shown by higher protein surface coverage and significantly more negative charges on the droplets. WPI-CMC stabilized emulsions showed smaller droplet size and had improved stability. Overall, increasing CMC concentration up to 0.1% enhanced the adsorption of protein at the interface and increased the negative charge of the droplets, resulting in smaller droplet size and more stable emulsions. Above 0.1% CMC, the droplet surfaces were

saturated with CMC as shown by little or no change in droplet size and ζ-potential, and protein surface coverage. Further addition of CMC would increase the viscosity of the continuous aqueous phase and/or form weak gel-like network. Consequently, the emulsions were more stable against creaming.

Although CMC molecular weight did not show significant difference on ζ-potential or droplet size of the emulsion, it significantly influenced protein adsorption as well as the flow behavior and the stability of the emulsions. Overall, higher M_w CMC contributed to more stable emulsions compared to lower M_w CMC according to the creaming index. This was likely due to combined effects of increased protein surface coverage on the droplets and viscosity of the emulsions. Viscosity effect strongly dominated at high CMC concentration with emulsions containing high M_w being the most stable. Complexation between WPI and CMC even at pH > pI provided improved the surface properties of the protein and enhanced the electrostatic as well as steric repulsion of the adsorbed layers, contributing to the stability of acidified WPI/CMC-stabilized emulsions.

5.2 Acid-induced gelation of heated soluble WPI-CMC complex

Acid-induced gels formed from WPI-CMC complex (biopolymers heated together) were firmer compared to those formed from polymer-CMC (biopolymer heated separately). Though microstructural analysis showed less phase-separated structure in gels from WPI-CMC complex, this difference was not shown in water holding capacity measurement. The only exception was at 0.05 wt% CMC 750k

where significant difference in WHC between WPI-CMC complex and polymer-CMC was observed. CMC molecular weight and biopolymer ratio were the major factors affecting the properties of acid-induced WPI-based gels. With different molecular weight of CMC, gel hardness showed significant different trends as a function of CMC concentration. When WPI-CMC complex was heated at higher initial protein concentration (9%), significantly higher WHC and gel hardness were shown compared to those from heated at 6% protein.

In conclusion, this study demonstrated that gelation properties of whey protein/CMC mixed systems can be improved by heated WPI-CMC soluble complex. Heating whey protein and CMC together to form the soluble complex promoted the interactions between the two biopolymers, which contributed to the reduced micro-phase separation with smoother and less porosity of gel structures, thus improving gel hardness and water holding capacity.

5.3 Overall benefits and future directions

Soluble WPI-CMC complexes have a great potential to be novel food ingredients due to their improved functional properties. Understanding different factors that affect their formation and functional properties could lead to the design of the complexes that are suitable for different applications. The study demonstrates that both unheated and heated soluble WPI-CMC complex can be used as a novel ingredient in food emulsion and cold-set food gels. Firstly, the improved stability of WPI-CMC stabilized emulsion may contribute to the stability and long-term shelf

life of emulsion-based products and acidified protein drinks. Secondly, the enhanced gel hardness and water holding capacity has its potential to be applied in yogurt, meat and other high protein low pH food products. We have shown here that molecular properties and biopolymer ratios are the major factors affecting the properties of the complexes. This could lead to future research on modification of polysaccharides to achieve certain molecular sizes in order to maximize their properties in complexation with proteins and in food applications.

APPENDIX

Table 1. Data of zeta-potential and droplet size for emulsions prepared with different M_w CMC.

CMC concentration (%)		Zeta-potential (mV)	Droplet size (μm)
Without CMC		-2.22±1.8	9.36±1.5
CMC 2,500k	0.04	-26.7±1.9	7.23±1.5
	0.08	-33.5±0.4	3.63±0.3
	0.1	-35.1±0.1	3.72±0.3
	0.3	-44.0±1.1	4.75±0.1
	0.5	-46.6±0.8	4.55±0.3
CMC 750k	0.04	-30.8±3.4	6.19±0.5
	0.08	-34.8±3.6	5.47±0.1
	0.1	-36.1±3.1	4.35±0.2
	0.3	-42.0±1.6	4.63±0.0
	0.5	-46.1±1.0	4.14±0.0
CMC 270k	0.04	-30.2±0.6	6.27±1.0
	0.08	-34.1±1.8	5.47±0.0
	0.1	-35.2±2.2	4.73±0.4
	0.3	-39.6±1.7	4.41±0.1
	0.5	-40.5±0.1	4.21±0.1

Table 2. Data of creaming index for emulsions prepared with different M_w CMC during 15-day storage.

CMC concentration (%)		Creaming index (%) day 5	day 10	day 15
Without CMC		52.4±4.5	57.1±2.4	61.0±3.6
CMC 2,500k	0.04	9.16±0.1	13.7±2.0	19.1±0.9
	0.08	0	0	0
	0.1	5.46±1.0	8.58±0.9	8.58±0.9
	0.3	6.86±1.0	11.4±3.1	16.8±2.0
	0.5	0	0	6.98±1.2
CMC 750k	0.04	46.2±0.0	53.1±1.1	57.7±1.1
	0.08	29.2±2.2	49.2±2.2	65.4±3.3
	0.1	22.3±1.1	24.6±4.4	54.6±3.3
	0.3	12.3±0.0	19.2±1.1	30.0±1.1
	0.5	10.4±1.6	20.0±4.4	30.8±8.7
CMC 270k	0.04	47.0±6.4	56.1±4.3	58.3±5.4
	0.08	36.6±3.9	61.8±1.7	64.1±1.5
	0.1	32.8±1.4	63.4±3.9	71.8±0.8
	0.3	26.7±0.8	66.4±0.4	67.2±1.4
	0.5	22.1±1.3	42.3±1.1	53.8±2.2

Table 3. Data of protein surface coverage of emulsions prepared with different M_w CMC.

CMC concentration (%)		Protein surface coverage (mg/m^2)
Without CMC		0.22±0.08
CMC 2,500k	0.04	0.84±0.00
	0.08	2.13±0.04
	0.1	1.54±0.23
	0.3	1.04±0.11
	0.5	1.17±0.01
CMC 750k	0.04	0.82±0.08
	0.08	0.78±0.19
	0.1	1.42±0.18
	0.3	1.25±0.01
	0.5	1.17±0.33
CMC 270k	0.04	0.49±0.04
	0.08	0.99±0.29
	0.1	1.18±0.21
	0.3	0.69±0.16
	0.5	1.23±0.10

Table 4. Data of initial force and water holding capacity (WHC) for gels from WPI-CMC complex and polymer-CMC.

CMC concentration (%)		Initial force (g)		WHC (%)	
		complex	polymer	complex	polymer
Without CMC			99.23.4		93.2±1.1
CMC 270k	0.01	137.9±13.7	105.8±16.0	93.7±0.2	93.2±0.4
	0.025	144.1±8.5	132.9±12.8	92.2±0.5	91.7±0.5
	0.05	158.8±18.0	125.1±15.6	80.0±9.8	67.5±2.6
	0.075	197.3±29.2	146.0±6.4	51.7±4.3	46.3±0.8
	0.1	205.7±20.0	178.4±20.5	35.5±2.5	33.2±1.3
	0.125	249.6±21.1	189.5±13.7	29.4±1.4	25.3±0.9
CMC 680k	0.01	149.8±30.8	139.5±10.0	93.9±1.6	92.9±0.4
	0.025	166.9±28.6	163.3±17.7	92.1±0.9	91.9±0.5
	0.05	179.3±31.9	196.5±18.0	78.6±3.5	80.2±5.1
	0.075	198.6±29.6	167.0±17.8	37.8±2.7	33.0±0.3
	0.1	200.4±47.2	108.0±12.1	29.5±0.8	26.1±0.8
	0.125	137.7±36.6	-	28.6±2.5	-
CMC 750k	0.01	159.9±10.4	131.6±17.5	93.2±1.5	93.6±0.2
	0.025	192.2±17.4	175.4±11.5	88.3±5.0	88.8±8.2
	0.05	249.8±7.7	194.9±7.6	74.4±10.6	44.6±1.3
	0.075	202.4±7.7	144.7±17.9	28.1±0.5	26.0±2.1
	0.1	54.7±16.6	37.7±13.9	28.7±1.1	28.3±0.3
	0.125	-	-	-	-

Table 5. Data of initial force and water holding capacity (WHC) for gels from 9%WPI-CMC complex.

CMC concentration (%)	Initial force (g)	WHC (%)
Without CMC	171.0±15.7	97.6±0.1
0.01	205.5±30.3	97.3±0.5
0.025	241.5±33.2	97.0±0.5
0.05	272.5±32.8	97.2±0.3
0.075	283.6±39.7	91.9±2.9
0.1	267.6±35.5	77.8±9.7
0.125	301.2±38.7	94.7±1.0

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