ABSTRACT

Title of Document: CHARACTERIZATION OF INTERACTION BETWEEN WHEY PROTEIN ISOLATE AND XANTHAN/CURDLAN HYDROGEL TO IMPROVE FREEZE-THAW STABILITY

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Syneresis, the liquid separation from a food product, is one of the major challenges in the frozen food industry. It is often accelerated by unintentional temperature fluctuations, i.e. repeated freezing and thawing during distribution, transportation, storage, and consumption. It has been demonstrated that the combination of xanthan and curdlan is capable of reducing syneresis up to five freeze-thaw cycles (FTCs) with relatively stable rheological and textural properties. The present study aimed at developing an effective mixture of whey protein isolate (WPI) and xanthan-curdlan hydrogel complex (XCHC) to minimize moisture migration over multiple FTCs. The addition of XCHC to WPI solution significantly reduced the syneresis of heat-induced gels, increased the storage modulus (G') of the gels, reduced the minimum

concentration of whey protein isolate required to form a gel, and minimized the discrepancies of G' in frequency sweep tests over 5 FTCs. By comparing the microstructure of mixed WPI-XCHC and pure WPI gels, it was found that XCHC served as a pore-forming agent, namely increasing the porosity, reducing the pore size, and consequently improving the gel's water retention over multiple FTCs. Results from dynamic rheological measurements showed that both G' and the gelation temperature of mixed WPI-XCHC complex were strongly pH-dependent. Moreover, the interactions between WPI and XCHC in aqueous solution were characterized. An edible coating solution containing the mixture of WPI, xanthan, and curdlan was tested on mushroom and green bell pepper. Fresh mushroom and green bell pepper samples treated with WPI-XCHC significantly prevented moisture migration after 10 days of frozen storage. The coating also decreased the changes of whiteness and greenness in mushroom and bell pepper, respectively, while significantly improved the firmness of bell peppers. Such information could provide useful guidelines when designing novel food products utilizing the unique properties provided by WPI-XCHC.

CHARACTERIZATION OF INTERACTION BETWEEN WHEY PROTEIN ISOLATE AND XANTHAN/CURDLAN HYDROGEL TO IMPROVE FREEZE-THAW STABILITY

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To my husband and family.

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List of Abbreviations

BF: Before Freezing

FTC: Freeze-Thaw Cycle

FTIR: Fourier Transform Infrared

G': Storage Modulus

G'': Loss Modulus

O.D.: Optical density

O.D.sub.280: The optical density measured at a wavelength of 260 nm

pI: Isoelectric Point

WL: Weight loss

WP: Whey Protein

WPC: Whey Protein Concentrate

WPI: Whey Protein Isolate

XCHC: Xanthan Curdlan Hydrogel Complex

Chapter 1: Introduction

1.1 Introduction

Food is a complex, multi-component system containing various ingredients such as proteins, lipids, carbohydrates, polysaccharides, minerals, and water. Among these components, proteins and polysaccharides are two key macromolecules that are responsible for the structural, mechanical, and other physicochemical properties in food systems, through their gelling, thickening, and surface-stabilizing functional properties (Kelly, 1995; Schmitt et al., 1998). A proper understanding of the interactions between proteins and polysaccharides, and controlling those interactions, enables food scientists to design products with a desired structure and texture (Turgeon et al., 2003).

One of the main applications of hydrocolloids in food systems is their ability to increase water holding capacity that could consequently improve freeze-thaw stability in frozen products. Texture instability remains the most significant challenge for frozen food products, especially with the inevitable post-production temperature fluctuations. Loss of moisture and changes in textural attributes often result in significant reduction of product quality (Franks, 1985). As the demand for ready-to-eat food items rises, a wide variety of frozen food products are constantly introduced to the markets. The U.S. market for frozen foods has grown to $28 billion over the past two decades as the penchant for eating "on the run" continues (Mintel, 2009). Therefore, to keep frozen foods from becoming junk foods (i.e., to retain their nutrients) it is critical to minimize syneresis, or expulsion of moisture from food,

which is caused by temperature abuse of the products. Various polysaccharides have been employed as stabilizers for that reason. For example, konjac, agar, curdlan, and carrageenan are used in emulsified meatballs (Hsu & Chung, 2000); xanthan and galactomannan are added as formulation aids to enhance the freeze-thaw properties of starch gels (Lo & Ramsden, 2000); and xanthan gum has been shown to be highly effective in reducing the syneresis in sweet potato starch gel (Lee et al., 2002). The combination of xanthan and curdlan is capable of eliminating syneresis for up to five freeze-thaw cycles while exhibiting stability in several rheological experiments (Williams et al., 2009).

Whey protein is an important source of functional protein ingredients in many food formulations. Whey proteins are very sensitive to heat, and heat treatment during the manufacture of whey protein affects the solubility and other functional properties of proteins. Therefore, to increase the applicability, the functional properties of whey protein need to be manipulated. Using polysaccharide and the formation of protein-polysaccharide complexes is one of the useful techniques to improve functional properties of whey protein (Mishra et al., 2001).

The interest in finding novel materials and techniques to develop value added structures is increasing in food industry. These structures can be used as the functional food ingredients in modification of the texture and mouthfeel of the foods; as formulation of meat analogues, fat replacer, edible films and coatings; and as an encapsulation device for bioactive compounds. In this area, biopolymer particles formed from proteins and polysaccharides can be ideal in the food industry for enhancing the stability and viability of the active food ingredients. However, despite

the broad improvements made in recent decades, polysaccharide and protein interactions in food hydrocolloids continue to be one of the most challenging topics to understand (Doublier et al., 2000; Turgeon et al., 2003; Ghosh & Bandyopadhyay, 2012).

Xanthan in combination with curdlan has been demonstrated by our research group to reduce syneresis and water loss, to a point where it is not detected and yields stable rheological and physical properties over freeze-thaw cycles when compared to curdlan that is combined with guar, locust bean, or k-carrageenan gums (Williams, 2011). To date, there is no research about the effect of xanthan curdlan hydrogel complex (XCHC) on other biopolymers. Real food systems are the combination of proteins and polysaccharides; therefore, studying the interaction of XCHC and whey protein, an important functional ingredient, can be useful in finding new applications for XCHC in the food industry.

1.2 Research Objectives

The ultimate goal of the main objectives of the present study is to develop a mixture of whey protein isolate (WPI) and XCHC to minimize moisture migration and improve freeze-thaw stability. Characterization was achieved using rheology, microscopy, and Fourier Transform Infrared (FTIR) spectrometry. Moreover, the characterization of the complexes between WPI and XCHC biopolymers over a pH range of 2-8, on the formation of the complexes, was investigated by using a spectrum of parameters including zeta potential, turbidity, and particle size. Finally, the efficacy of a mixed system (containing whey protein, xanthan, and curdlan) as an edible coating was applied to mushroom and green bell pepper, and the effects of the

system on the moisture migration, weight loss, color, and texture of the thawed-frozen vegetables were examined. In order to achieve this goal, there were four specific objectives, where the subsequent chapters following the literature review are presented as separate manuscripts:

Objective 1*:* Investigate the effect of xanthan curdlan hydrogel complex on syneresis and rheological properties of heat-induced whey protein isolate gel over multiple freeze-thaw cycle

Objective 2*:* Investigate the effects of pH on gelation temperature and elasticity of mixed gels containing WPI and XCHC

Objective 3: Study the interactions between WPI and XCHC in aqueous solutions with different pH values and ratios of WPI: XCHC

Objective 4*:* Explore efficacy of the mixed whey protein isolate, xanthan, and curdlan as an edible coating in frozen-thawed mushroom and green bell pepper to investigate quality aspects, including weight loss, color, and texture

Chapter 2: Literature Review

2.1 Food Hydrocolloids: Applications and Challenges

Food hydrocolloids, high molecular weight hydrophilic biopolymers, are used as the functional ingredients in the food industry in order to control the microstructure, texture, flavor, and shelf-life of the product; hydrocolloids are either protein or polysaccharide (Dickinson, 2003). Food hydrocolloids are obtained from different sources, and the major sources are botanical, algal, animal, and microbial sources. Improving the texture and pouring properties of many food products is impossible without utilizing the ability of food hydrocolloids to control water distribution; as a result, they have been the bases of the revolution in food fabrication. Hydrocolloids contain a large number of hydroxyl (-OH) groups, which increases their affinity to bind water molecules, thus giving them a high hydrophilic character (Saha & Bhattacharya, 2010).

Proteins are famous for their emulsification and foaming properties, and polysaccharides for their water-holding and thickening properties. Both proteins and polysaccharides are important in the structural and textural characteristics of foodstuffs by using their aggregation and gelation behavior (Dickinson, 2003). Hydrocolloids function as thickening and gelling agents, texturizers, stabilizers, and emulsifiers (Dickinson, 2009; Saha & Bhattacharya, 2010). They also have many applications in edible coatings and flavor release (Gennadios et al., 1997; Kampf & Nussinovitch, 2000; Varela & Fiszman, 2011), and in increasing freeze-thaw stability of the food products (Defreitas et al., 1997; Garcio-Ochoa et al., 2000;

Muadklay & Charoenrein, 2008). The formulation of fat-free or fat-reduced products is remarkably dependent on the functional properties of the hydrocolloids (Luruena-Martınez et al., 2004; Laneuville et al., 2005; Sahan et al., 2008).

The practice of combining different hydrocolloids to adjust the desired flow behavior and textural properties through the synergistic interactions between hydrocolloids is widely used in the food industry. Using the mixture of hydrocolloids offers the possibility of novel functionalities, reduces the levels of hydrocolloids, and, as a result, can contribute to cost reduction (Williams et al., 1991; Katzbauer, 1998).

2.2 Xanthan Gum

2.2.1 Sources and Structure

Xanthan gum (Figure 2.1) is an anionic microbial polysaccharide secreted by the *Xanthomonas campestris*. It is a gram negative, yellow-pigmented bacterium which can be found on the leaves of the Brassica vegetables such as cabbage (Sworn, 2000). Xanthan gum is one of the major commercial biopolymers produced with an annual worldwide production of 30,000 tons, corresponding to a market of $408 million (Kalogiannis et al., 2003). It is soluble in hot or cold water and the molecular weight of xanthan gum can reach up to 6 MD, which makes it capable of creating a high viscosity solution even at very low concentrations. It is very resistant to enzymatic degradation and stable over a wide range of pH and temperature. After dextran, xanthan gum is the second microbial polysaccharide to be commercialized (Palaniraj & Vijayakumar, 2011).

Xanthan was discovered in 1963 at the Northern Regional Research Center (now called The National Center for Agricultural Utilization Research) of the United States Department of Agriculture (USDA). In 1965, it was approved by the United States Food and Drug Administration (FDA) for use in food additives and became the most used thickening agent in food products due to its stability over a wide range of temperatures and pH levels (Margaritis et al., 1978). The backbone of xanthan is a β-(1-4)-D-glucose, which is the same as cellulose. Every alternate glucose reside has a trisaccharide side chain consisting of two mannose residues with a glucuronic acid residue between them. Xanthan gum has an excellent stability under adverse conditions due to the structure of xanthan gum. The side chains wrap around the backbone, so they can protect the labile β - (1→4) linkages from attack (Palaniraj & Jayaraman, 2001). Xanthan gum solutions are highly pseudoplastic. The viscosity of solution containing xanthan gum decreases as the shear rate increases. This characteristic of the xanthan gum can be attributed to its helical structure (Kalogiannis et al., 2003). Once the shear is removed, the starting viscosity is recovered; this might be due to the ability of xanthan molecules to form aggregates through hydrogen bonds and polymer entanglement (Sworn, 2000). Xanthan solutions are highly ordered, entangled, stiff molecules at low shear rates. Increasing the shear rate will lead to the interruption of aggregates, and individual polymer molecules align in the direction of the shear force, which results in the pseudoplastic conditions (Sadar, 2004).

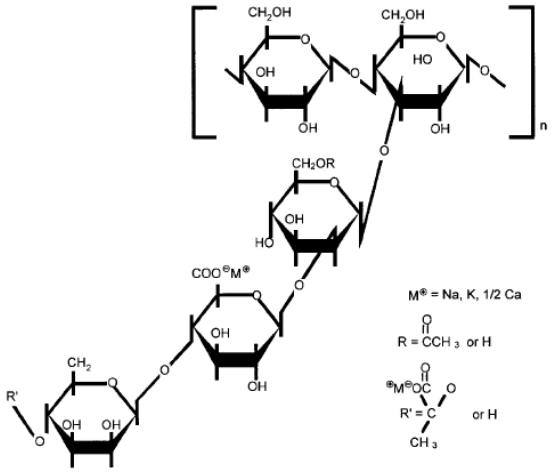


Figure 2.1. Structure of xanthan gum (Sworn, 2000).

2.2.2 Applications in Food and Other Industrials

The major application of xanthan gum is in the food industry: as a thickener, stabilizer, emulsifier, suspending agent, and foam enhancer (Nussinovitch, 1997; Garcia-Ochoa et al., 2000; Gumus et al., 2010). Xanthan gum is capable of synergistic interactions with other gums such as carrageenan, guar, and locust bean gum. Therefore, the use of a combination of xanthan gum and a secondary polysaccharide is widespread in the food industry (Williams et al., 1991; Sandolo et al., 2010; Harding et al., 2011). In milk and dairy products, the combination of xanthan and other gums is very common. They can provide optimal viscosity and long-term stability, improve heat transfer during processing, enhance flavor release, and control the growth of ice-crystal (Sharma et al., 2006; Rosalam & England, 2006). Xanthan can improve the freeze-thaw stability over multiple freeze-thaw cycles by controlling viscosity and increasing the water holding capacity (Sworn, 2000; Lo & Ramsden, 2000; Garcia-Ochoa et al., 2000; Mandala, 2005).

The special rheological properties of xanthan has made it suitable for the enhanced oil recovery (EOR) application. Xanthan gum is used as a lubricant, emulsifier, and mobility-control agent in the oil drilling-industry (Katzbauer, 1998). In the pharmaceutical and cosmetic industries, xanthan gum is used as a thickening and suspending agent.

2.2.3 Rheological Properties

Studies on the structure and conformation of xanthan gum showed that it has many unique solution properties. In aqueous solution, xanthan molecules form a semiflexible wormlike structure with a weak-gel network as a result of the intermolecular interactions (Milas & Rinaudo, 1986; Rochefort & Middleman, 1987; Lee & Brant, 2002). These intermolecular association, such as hydrogen bonding, electrostatic and hydrophobic interactions, are responsible for the unique rheological properties of xanthan gum (Rochefort & Middleman, 1987).

Xanthan is an anionic polysaccharide containing negatively charged groups of glucuronic acid and pyruvate in its side chain (Richardson & Ross-Murphy, 1987). Rheological parameters depend on the ionic strength, pH, temperature, and solvent quality. Increasing the ionic strength in xanthan solution increases hydrodynamic radius, and consequently the high viscosity of xanthan solution remains stable (Chen & Shepperd, 1980; Southwick et al., 1982). At low salt concentrations (up to 0.05 M) the changes of viscosity and dynamic properties in xanthan gum solutions are not significant (Southwick et al., 1982). Xanthan gum has the ability to retain its viscosity until a specific melting temperature is reached. At this temperature, the viscosity falls notably due to a reversible change in molecular conformation (Sworn, 2000).

2.3 Curdlan

2.3.1 Sources and Structure

Curdlan is a neutral extracellular microbial polysaccharide produced commercially from the fermentation of glucose by the microorganism *Alcaligenes faecalis* var. *myxogenes*. It is a linear homopolymer of D-glucose with ß (1, 3) glucosidic linkages (Figure 2.2), and capable of forming a gel by heating (Nakao, 1997; Nishinari & Zhang, 2000). Curdlan was approved by the U.S. Food and Drug Administration (FDA) in 1996 as a direct food additive (Funami, 1999). It has the ability to form an elastic gel, which makes it unique as a food additive. In addition, it is believed to have strong bioactivities (Nishinari & Zhang, 2000). Curdlan forms two types of heat-induced gels including thermo-reversible low-set gel and thermo-irreversible high-set gel. The low-set gel is obtained when the aqueous solution is heated between 55 and 60ºC and then cooled below 40ºC, whereas the high-set gel is obtained when the aqueous solution is heated above 80ºC; and, it is stable at low temperatures such as freezing and at high temperatures as in retorting (Funami, 1999). Although curdlan is a linear homopolymer chain, it is capable of forming complex tertiary structures, which can be attributed to the intra- and intermolecular hydrogen bonding (Nishinari & Zhang, 2000).

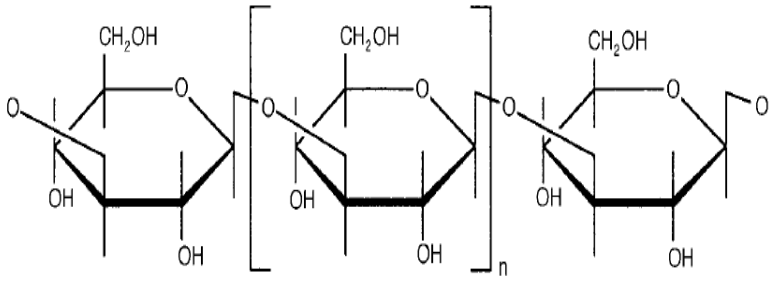


Figure 2.2. Structure of curdlan (Nishinari & Zhang, 2000).

2.3.2 Applications in Food and Other Industrials

Curdlan, a tasteless, odorless, and colorless microbial gum, has many applications in the food industry. Its unique property is its ability to form a firm, resilient, and thermo-irreversible gel when heating above 80°C. The gel does not melt even above the temperature of 100°C, which makes it appropriate to use during processes such as thermal sterilization (Miwa et al., 1993). Curdlan can form a gel even in the presence of large amounts of lipids in the food product formulation.

Adding curdlan to the noodle dough softens the noodle and prevents soluble ingredients from leaking out, leading to a clear soup broth. The texture of thawed-frozen tofu containing curdlan is smooth and more acceptable (Nishinari & Zhang, 2000). Curdlan can improve the elasticity of the surimi products, alter the texture and the shape retention of the frozen desserts, and improve the water-holding capacity of the meat products. One of the main purposes of using curdlan in food products is to inhibit ice crystallization and moisture migration, as it can bind additional water. The study of curdlan-based fat mimetics in nonfat sausages showed that curdlan was effective as a fat replacer in such products (Funami et al., 1998). Curdlan is capable

of forming hydrogel complexes with other polysaccharides. Therefore, it can be used to increase retention or absorption of moisture and other ingredients (Lo et al., 2003).

Compared to other microbial polysaccharides (xanthan and gellan), applicability of curdlan in the U.S. market is limited, mainly because it has lower viscosity than xanthan gum and less gel formation capacity than gellan (Sadar, 2004). Recently, the positive effects of curdlan in the biomedical applications particularly in the field of immunology were reported. Curdlan and its derivatives were capable of inhibiting tumor growth, and a sulfated derivative of curdlan showed anti-HIV effects (Zhan et al., 2012). It also has some applications in the field of controlled drug delivery (Kanke et al., 1995).

2.3.3 Rheological Properties

The study of rheological and gelling properties of curdlan aqueous solutions has showed shear-thinning properties (Funami et al., 2000; Lo et al., 2003). Curdlan aqueous solutions demonstrated some unique characteristics that distinguished it from other hydrocolloids. It can form thermo-reversible gels by heating to 60°C, adding Ca^{2+}or Mg^{2+}, or neutralizing an aqueous alkaline solution. It is also capable of forming strong, irreversible gels when heated above 80°C (Hirashima et al., 1997; Funami et al., 2000, 1999; Lo et al., 2003).

As mentioned before, gels formed at higher temperatures are thermo-irreversible, and gels formed at lower temperatures are thermo-reversible. The effect of heating rate on the gelling properties of curdlan aqueous dispersions was investigated, and the results showed that a rising heating rate would lead to an increase in storage modulus (G'), which was attributed to the increase in thermo-

reversible component or the number of junction zones between curdlan molecules (Funami et al., 2000). As gel concentration increases, the intermolecular cross-linking and apparent viscosity rises, leading to a reduction of mobility in polymer chains and slower release rates (Lo et al., 2003).

2.4 Xanthan-Curdlan Hydrogel Complex

The texture stability of different hydrogel complexes containing curdlan and a secondary biopolymer (i.e., κ-carrageenan, guar, locust bean, and xanthan gums) over multiple freeze thaw cycles (FTCs) was studied. Results showed that xanthan/curdlan hydrogel had the highest freeze–thaw stability in terms of syneresis, heat stability, and adhesiveness. Xanthan curdlan hydrogel could eliminate syneresis for up to five FTCs, while it showed stability in several rheological experiments (Williams, 2011).

2.5 Whey Protein

2.5.1 Structure and Properties

Whey is a by-product of the cheese-making process and whey proteins are technically defined as those that remain in the milk serum after coagulation of casein at a pH of 4.6 and a temperature of 20ºC (Dangaran et al., 2009). Whey proteins are widely used as a food ingredient due to their nutritional and useful functional properties. They are multifunctional ingredients providing properties such as gelation, water binding, solubility, foaming, viscosity, and emulsification for foods. One of the unique properties of whey protein is its good solubility in water over a wide range of pH (Burrington, 2006).

Gelling is one of the most important functional properties of whey proteins, the result of an aggregation process that is induced by changing the conditions (usually increasing the temperature). ß-lactoglobolin is the major whey protein that participates in gelling, comprising 10% of the total milk protein or about 58% of the whey protein. It contains 162 amino acids with a molecular weight of about 18.3 kDa. The second most frequent protein in whey is α-lactalbumin that forms about 2% of the total milk protein (about 13% of the total whey protein). It is a small molecule (Mw=14.14 kDa) with 123 amino acids.

The aggregation of whey proteins occurs in the pH range of 4.2 to 5.2, which is the range for α-lactalbumin and ß-lactoglobolin isoelectric points. Three phenomena are involved in the aggregation of globular proteins: conformational changes, chemical reactions, and physical interactions (Verheul & Roefs, 1998). At the pH of isoelectric point (pI), the net charge of the whey protein molecules is at the minimum level; at the pH below the pI, it is positively charged; and above the pI, it is negatively charged. The properties of whey protein gels differ from viscous fluids, curds to rubbery gels. Gels formed by milk proteins are usually irreversible and occur because of enzymatic reactions, heat-induced reactions, cation (calcium) interactions, or from combined reaction mechanisms. Aggregation and gelling properties of whey protein gels depends on the pH, salt concentration, and the type of the salt or heating conditions (Zayas, 1997). When the electrostatic repulsions are strong, gels from globular proteins are transparent with a fine-stranded structure, whereas they are opaque with a coarse, lumpy, particulate structure in conditions of weak electrostatic repulsions (Verheul & Roefs, 1998).

2.5.2 Applications

There are many industrial applications of whey products in the food industry (Table 2.1). Whey protein has nutritional uses in protein uptake, especially for athletes who use WP to increase their muscle mass and improve their performance. It also has some applications in cosmetics, such as skin protection and antimicrobial properties.

Table 2.1. Applications of whey protein in food industry. Adopted from Cayot & Lorient, 1997.

Industrial applications	Functional properties expected	Protein used
Bread making	Water holding	WPC or WPC+ Caseinates
Biscuit manufacturing	Fat dispersibility	WPI
Breakfast cereals	Emulsion stabilization	WPI, copercipitates
	Overrun of foam	Whey
	Gelling properties	
	Browning	
	Aroma enhancement	
Pasta	Binding and texturing effect	Copercipitates
	Browning	
Confectionary	Emulsion manufacturing	WPC+ hydrolyzed caseinates
Chocolate	Overrun of foam	WPC
	Browning, aroma	Whey
	Antioxidizing effect	Copercipitates
Ice cream	Emulsion stabilization	WPC+ Caseinates and total milk proteins
	Overrun of foam	
	Gelling properties	
Meat products	Emulsion making	WPC, WPI alone or in mixture with caseinate
	Water holding, adhesive or binding properties	
Sauces and soups	Emulsion stability, water holding	WPC+ caseinates + egg yolk
Ready-to-eat foods		
Milk products	Emulsion stability	Caseinates
(Cheese, yogurts)	Water holding	WPC + Caseinates
	Gelling properties	WPI
Alcoholic beverages	Cream stabilization	WPC + Caseinate
	Cloudy aspects	WPC or WPI

2.5.3 Whey Protein Isolate-based Edible Films and Coatings

An edible film or coating is any type of the material, which is used to coat or wrap foods in order to extend the shelf life of the product. They might be eaten with food, with or without further removal. They prevent moisture losses and selectively control the gas transfer (such as oxygen, carbon dioxide, and ethylene) in the food product. They can also be manipulated to carry flavor and provide surface sterility and prevent loss of other important components. The thickness of edible films and coatings is usually less than 0.3 mm (Pavloth & Orts, 2009). The major components of edible films and coatings are polysaccharides, proteins and lipids. Polysaccharides have good gas barrier properties, but their water vapor permeability is low because they are highly hydrophilic. The interest in using whey protein in edible films and coatings has increased. Whey protein-based edible films are transparent, flexible, colorless, odorless, impermeable to oil, a good oxygen barrier, and heat sealable (McHugh & Krochta, 1994; Yoo & Krochta, 2011). The use of whey protein isolate as an edible film and coating, with different functionalities and applications, has been studied previously (Chae & Heo, 1997; Letendre et al., 2002; Kokoszka et al., 2010; Jiang et al., 2010). Edible films from whey protein isolates exhibit moderate moisture barrier and excellent oxygen barrier properties (Gounga et al., 2007). Heat-denatured whey proteins produce transparent and flexible water-based edible coatings with excellent oxygen, aroma, and oil barrier properties at low relative humidity. However, since whey proteins are hydrophilic, they are known to have poor moisture barrier properties. Water insoluble proteins, such as corn zein, produce insoluble coatings, whereas water-soluble proteins produce soluble coatings. Their solubility is

dependent on the protein and the conditions of coating formation and treatment. Whey protein isolate produces water-soluble coatings, but heat denatured solutions of whey protein isolate produce coatings in which the protein is insoluble (Perez- Gago et al., 1999; Dangaran et al., 2009).

2.6 Protein-Polysaccharide Complexes

Proteins and polysaccharides are the fundamental functional ingredients present in many food systems, contributing to the shelf life, structure, texture, and stability of food by their thickening, gelling, and surface properties. Generally, in the food industry, proteins are used for their emulsifying properties; polysaccharides for their stabilizing properties. Coupling the two biopolymers allows us to combine these properties (Doublier et al., 2000; Dickinson, 2008).

The interest for the study of protein–polysaccharide interactions has grown markedly in the food industry, creating new opportunities for the design of new ingredients and products with unique functional, structural, and sensory properties. Controlling or manipulating these macromolecular interactions is a leading factor in developing novel and fabricated formulations in the food industry (Tolstoguzov, 1997). Protein-polysaccharide complexes can exhibit improved functionality over proteins and polysaccharides alone. Internal and external factors such as pH, ionic strength, protein to polysaccharide ratio, total concentration of biopolymers, the charge of the proteins and polysaccharides, quality of solvent, and molecular weight affect the formation and stability of complexes (Glahn & Rolin, 1995; Weinbreck et al.,, 2004; de Kruif et al., 2004).

The application of protein-polysaccharide complexes in the food formulations (such as fat replacer, texturing agent, and meat analogues), food packaging, and edible films formation has been patented in many previous studies (Chen & Soucie, 1986; Chen et al., 1988; Lawson & Lin, 1991; Bakker et al., 1994). The distinct properties of protein-polysaccharide complexes enables us to use them in the purification of proteins or polysaccharides, in microcapsule formation, and in the design of biomaterials (Schmitt et al., 1998).

Generally, interactions between proteins and polysaccharides in aqueous media can result in one- or two-phase systems. Proteins and polysaccharides can be compatible or incompatible in aqueous solutions. The nature of interactions, either repulsive or attractive, will lead to different behaviors such as co-solubility, segregation (Incompatibility), and associative phase separation (Complex conservation) (Schmitt et al., 1998).

Complex coacervation is a spontaneous liquid-liquid phase separation that occurs when two oppositely charged polymers are mixed. The mixture separates into two phases: the lower phase containing the protein-polysaccharide complex (complex coacervate) and the upper phase containing mainly the solvent (supernatant or equilibrium phase). Complex coacervates have a broad range of applications, which is due to the fact that they are biodegradable, have high nutritional and functional properties, and originate from the biological sources. One of the most important industrial applications of protein-polysaccharide coacervates is microencapsulation. It is also a cheap and powerful method in the purification of macromolecules.

Coacervates are used as food ingredients, such as fat replacers, meat analogues, and edible films and packaging (Weinbreck et al., 2004).

Whey proteins (WP) are widely used in the food industry for their well-known high nutritional and functional characteristics, which are related to the structure and biological functions of these proteins (de Wit, 1998; Turgeon & Beaulieu, 2001). One of the most important functional properties of WP is gelation. Many factors, such as concentration, temperature, pH, ionic strength, heating rate, and the presence of specific ions affect the gelation of WP (Hines & Foegeding, 1993; Mulvihill & Kinsella, 1987). Furthermore, manipulating the functional properties of WP is needed to increase the applicability of whey protein. One of the methods to achieve this purpose is the formation of complexes of WP with polysaccharides (Mishra et al., 2001). To date, many researchers have studied the mixture of whey protein and different types of polysaccharides to find synergistic effects of biopolymers existing in food systems (Mleko et al., 1997; Bryant & McClements, 2000; Bertrand & Turgeon, 2007; Ibanoglu, 2002; Perez et al., 2009).

Functional properties, such as foaming, gelation, emulsification, and solubility of whey protein concentrate were improved when combined with pectin (Mishra et al., 2001). The interaction of whey protein concentrate and two polysaccharide (sodium alginate, and k-carrageenan) in the aqueous phase was studied, and results showed that the interactions have an effect on their foaming behavior and on the adsorption of mixed systems at the air–water interface (Perez et al., 2009). Depending on the pH, xanthan gum can have a synergistic or antagonist effect when added to WPI, leading to different gel microstructures (Bertrand &Turgeon, 2007). Table 2.2

shows the summery of the protein-polysaccharide systems that have been studied in the food industry, along with the experimental techniques used in these mixed biopolymer systems.

Table 2.2. Experimental techniques applicable for studying protein-polysaccharide complexes.

Systems	Purpose of study	Experimental techniques	References
β-lg/acacia gum	Study the stability and microstructure of β-lg/acacia gum coacervates	Confocal Scanning Laser Microscopy (CSLM), Diffusing wave spectroscopy(DWS)	Schmitt *et al.*, 2001a
β-lg/k-Carrageenan	Investigate the rheological behavior	Rheology, Differential Scanning Calorimetry (DSC)	Ould Eleya & Turgeon, 2000
β-lg/pectin	Develop an ITC method to study the interactions between the food macromolecules	Isothermal Titration Calorimetry (ITC)	Girard *et al.*, 2003
β- lg/pectin	Characterization of biopolymer particles based on thermal treatment of β- lg /Pectin electrostatic complexes	Turbidity, Dynamic light scattering, Electrophoresis, Lowry assay	Jones *et al.*, 2009
β-lg/sodium Alginate	Characterization of β-lg/sodium Alginate interactions	ITC, Turbidity, z-potential and particle size measurements	Harnsilawat *et al*., 2006
β-lg/tragacanthin	Study of complex coacervation between β-lactoglobulin and tragacanthin (soluble part of gum tragacanth)	Static & dynamic light scattering, Turbidity	Mohamadifar *et a*l., 2007
Whey Protein Isolate/carrageenan	investigate the complex formation of WPs and CG as a function of pH and ionic strength	Turbidimetric Titration, NMR, Conductivity	Weinbreck *et al.*, 2004
Whey Protein Isolate/k-carrageenan	Acid gelation properties of complex coacervates of WPI/k-Car	Zeta potential , Rheology, Turbidity	Mounsey, 2008
Whey Protein Isolate/k-carrageenan	Influence of shearing during heating on the phase distribution and rheology of k-car and WPI mixture	Rheology, Phase diagram, SLCM	Gaaloul *et al.*, 2009
Whey Protein Isolate/k-Carrageenan Whey Protein Isolate/ pectin	Improvement of the WP gels	CLSM, Syneresis, Texture Analysis	Turgeon & Beaulieu, 2001

Systems	Purpose of study	Experimental techniques	References
Whey Protein Isolate/gelatin	Study of the Physical Properties of WPI/Gelatin Composite Films	FTIR, Zeta Potential and Particle Size, SEM, DSC	Jiang *et al.*, 2010
Whey Protein Isolate/gum Arabic	Characterization of WP/GA coacervates	Rheology, HPLC, NMR,	Weinbreck *et al.*, 2004
Whey Protein Isolate/gum Arabic	Rheological behavior of whey protein stabilized emulsions in the presence of gum Arabic	Rheology	Ibanoglu, 2001
Whey Protein Isolate/methylcellulose Whey Protein Isolate/sodium alginate	Comparison of physical properties of the WPI-PS blended films with WPI or PS film alone	Mechanical tests, DSC	Yoo & Krochta, 2011
Whey Protein/pectin (LMP)	Study the effects of added calcium on WP/LMP	Rheology, CLSM, Texture Analysis	Beaulieu et *al.*, 2001
Whey Protein Isolate/xanthan	The effect of P/PS and microfluidization on WPI-xanthan	optical microscopy, computer-aided image analysis, Rheology	Laneuville *et al.*, 2000
Whey Protein Isolate/xanthan	If the application of cold-setting whey protein ingredients could be extended by making use of beneficial Pr-Ps interactions.	Turbidity, Rheology	Bryant & McClements, 2000
Whey Protein Isolate/xanthan	Investigate the gelation of WPI/ xanthan under different heating rates	Rheology, Uniaxial compression	Ould Eleya & Gunasekaran, 2005
Whey Protein Isolate/xanthan	Improvement of the WPI gels	Rheology, CLSM	Bertrand &Turgeon, 2007
Whey Protein Concentrate/k-Carrageenan Whey Protein Concentrate/sodium alginate	Interaction between WPC and K-Car,SA	Size-exclusion HPLC, DSC, Fluorescence spectroscopy	Perez *et al.*, 2009
Whey Protein Concentrate/alginate Whey Protein Concentrate/pectin Whey Protein Concentrate/carrageenan Whey Protein Concentrate/konjac	Study the physical properties of protein-polysaccharide–based edible films	Mechanical tests	Coughlan *et al.*, 2004
Whey Protein Concentrate/pectin	Evaluate the functional properties of WP/pectin mixture	Gel filtration profile, Syneresis	Mishra *et al.*, 2001
(Milk Protein+ k-Car) /LBG, guar gum, xanthan	The effect of k-carrageenan on phase separation between milk proteins and polysaccharide mixtures	Rheology, TEM, Phase Diagram	Thaiudom & Goff, 2003

Chapter 3: Influence of Xanthan-Curdlan Hydrogel Complex on Freeze-Thaw Stability and Rheological Properties of Whey Protein Isolate Gel over Multiple Freeze-Thaw Cycle

3.1 Introduction

Well known for their functional and nutritional properties (de Wit, 1998), whey proteins (WP) are widely used as a food ingredient due to those nutritional and useful functional properties. Gel formation is one of the most important functionalities of WP, and heat-induced gelation is one of the most common mechanisms utilizing WP gels to modify texture (Ziegler & Foegeding, 1990; Foegeding et al., 1998).

Two main steps are involved in the gelation process: first, increasing inter-molecular interactions; second, the aggregation of molecules into a gel network. In globular proteins, unfolding is required before gelation, and a critical balance between attractive and repulsive forces is required for the formation and stabilization of the gel network (Ziegler & Foegeding, 1990). An excess in attractive forces results in a precipitate, whereas extreme repulsive forces produces soluble aggregates. The most typical mechanism involved in forming WP gels is heat-induced protein denaturation (Foegeding et al., 1998). WP have a lot of sulfhydryl amino acid resides that allow them to form intermolecular covalent bonds during heating. The stability of the WP gels is related to the disulfide bonds and sulfhydryl groups. Intermolecular disulfides appear to be important to the elasticity of WP isolate gels (Zayas, 1997).

The first step in WP isolate (WPI) heat-induced gelation is the weakening and breaking of hydrogen and sulfide bonds and breakdown of the native protein

conformational structures. The second step is the polymerization of dissociated protein molecules and formation of a three-dimensional protein structure with a large amount of solvent immobilized through intermolecular disulfide, hydrophobic and ionic bonds. In protein gels, an ample portion of the water is bound by proteins, but most of the water is physically entrapped within the three-dimensional gel network (Zayas, 1997).

One of the main applications of WP in the food industry is in the formulation of frozen products such as frozen desserts and frozen yogurts. WP can modify the structural, textural and organoleptic properties of food, resulting in improved consumer acceptance of the food product (Rich & Foegeding, 2000). As the demand for ready-to-eat food products increases, a wide range of frozen food products are introduced into the market. Maintaining frozen food products in a constant and ideal frozen state is the most important challenge in frozen food products, especially with inevitable post-production temperature fluctuations and repeated freeze–thaw cycles (FTC) during the supply chain. In the freezing process, the moisture in the food transforms into ice which often leads to physical stress to the food matrix. In a thawed frozen product, the moisture can easily separate from the matrix and cause softening of the texture, drip loss, and decreasing overall quality of the product (Rahman, 1999). Loss of moisture and changes in textural attributes often result in significant reduction of product quality, leading to changes in syneresis and related rheological properties (Lee et al., 2002). Therefore, formulating and developing high quality frozen products which can be maintained throughout the manufacturing process, distribution, and storage is the most critical issue in the frozen food industry.

On the other hand, hydrocolloids are often used in food systems to increase water-holding capacity that could consequently improve freeze-thaw stability in frozen products. Using a proper amount of hydrocolloids can improve desirable textural properties and the stability of frozen products. The effect of hydrocolloids on freeze-thaw stability for different types of gels have been studied. Alginate, guar gum, and xanthan were highly effective in reducing the syneresis of sweet potato starch gel over 5 FTCs (Lee et al., 2002). Xanthan gum was capable of reducing syneresis in tapioca starch gel after 5 FTCs (Muadklay & Charoenrein 2008). Curdlan, having the ability to bind additional water, is used in food products to inhibit ice crystallization and moisture migration. It has showed positive effects on the texture of thawed frozen tofu (Nishinari & Zhang, 2000).

Williams et al. (2009) reported that xanthan curdlan hydrogel complex (XCHC) (1% w/v) could eliminate syneresis for up to five FTCs while showing stability in several rheological experiments. To date, there is no research about the effect of XCHC on other biopolymers. Real food systems are the combination of proteins and polysaccharides; therefore, studying the interaction of XCHC and WP, an important functional ingredient, can be useful in finding new applications for XCHC in the food industry.

The objective of the present study is to investigate the effect of XCHC on WPI gel and to develop a mixture of WPI-XCHC to minimize moisture migration and improve freeze-thaw stability over multiple FTCs. Five FTCs were conducted to simulate the possible temperature abuses of a frozen product from (1) processing plant to warehouse, (2) warehouse to distributor, (3) distributor to retail, (4) retail to

consumer, and (5) at home where the consumer may thaw and refreeze the product.

3.2 Materials & Methods

3.2.1 Materials

Odorless, fine, free-flowing white powder curdlan containing a minimum of 90% β-D-glucan and with a maximum of 10% water was used (Takeda Vitamin & Food USA, Orangeburg, NY). Xanthan (TICAXAN®) was kindly supplied by TIC Gums (Belcamp, MD); whey protein isolate (Hilmar 9400) by Hilmar ingredients (Hilmar, CA). The whey protein isolate used in this study consisted of 93.4% protein (% dry basis), Lactose (0.2%), fat (0.6%), moisture (4%), and ash (2.6%).

3.2.2 Preparation of Solutions

3.2.2.1 Xanthan-Curdlan Hydrogel Complex

To prepare xanthan curdlan hydrogel complex an equal amount of each of the biopolymer powders was weighed and dry blended at ambient temperature and gradually poured to deionized water under constant stirring. Gum solutions were agitated for 15 minutes to achieve homogeneous and lump free aqueous solutions. The biopolymer solution was magnetically stirred while gradually being heated on a heat plate until the temperature of the solution reached 90°C. After cooling to room temperature, gum solutions were covered and refrigerated overnight at 4°C to allow complete hydration. Two different solutions containing 0.5% (w/v) and 1% (w/v) of xanthan and curdlan were prepared.

3.2.2.2 Whey Protein Isolate Solution

Whey protein stock solutions 10% (w/v) were prepared by slowly dissolving the dried powder in deionized water agitated with a magnetic stirrer for 2 hours at room temperature to ensure complete dissolution. Solutions were then stored overnight at 4°C for complete hydration.

3.2.2.3 Mixtures of WPI and XCHC

Mixed solutions were prepared by mixing equal amounts of WPI solution (10%) and XCHC (0.5% and 1% (w/v)), the pH of mixtures were adjusted to 7 using 0.1N NaOH before agitating for 5 minutes at room temperature. In order to eliminate air bubbles from the solutions and obtain well-mixed samples, mixtures were centrifuged at 3000 rpm (1318×*g*) for 20 minutes at 20°C using a Beckman Model TJ-6 centrifuge (Beckman Coulter, Palo Alto, CA). Mixtures were poured into 50 mL test tubes and placed in a 90°C water bath for 30 minutes. Three different ratios of WPI: XC (5:0; 5:0.25; 5:0.5) were studied. Ratio of WPI: XC (5:0) was prepared by mixing equal amounts of WPI (10%) and deionized water as a control sample. Mixtures were cooled to room temperature and refrigerated overnight at 4°C before subjected to FTCs.

3.2.3 Methods

3.2.3.1 Freeze-Thaw Cycle

Mixtures of WPI and XCHC were subjected to one, two, three, four, and five freeze-thaw cycles. For each FTC, samples in the test tubes were frozen at -18°C for 18 h and then thawed at room temperature (25± 1°C) for 6 h. Syneresis and dynamic

rheological measurements were conducted before freezing and after each FTC to investigate the stability of biopolymer mixtures.

3.2.3.2 Syneresis Measurements

Syneresis of the mixtures of WPI and XCHC was determined before freezing and after each FTC by centrifuging the samples at 3000 rpm (1318 × *g*) for 20 min in a Beckman Model TJ-6 Centrifuge (Beckman Coulter, Palo Alto, CA). Syneresis was measured by the following equation:

$$\text{Syneresis (\%)} = [(W_a - W_b)/ W_a] \times 100$$

Where W_a is the initial weight and W_b is the weight of centrifuged sample. It should be noted that, the amount of syneresis, were reported in two steps: first syneresis after 6 h thawing (without centrifugation), and second additional syneresis by centrifugation of samples at 3000 rpm for 20 minutes.

3.2.3.3 Dynamic Rheological Measurements

Dynamic rheological measurements of the biopolymers mixtures were conducted using an AR2000 Rheometer (TA Instruments, New Castle DE) with a 40-mm steel truncated cone (2°) geometry with a truncation gap of 52 μm before and after each of the FTCs. Each sample was transferred to the rheometer plate at the desired temperature (20°C), and left standing for 2 minutes to allow structure recovery and temperature equilibration. The dehydration of the samples was limited by using a solvent trap. In amplitude sweep tests, the strain was increased from 0·01 to 10% at a constant frequency of 1 Hz to obtain the linear viscoelastic region (LVR). The storage (G') and loss (G'') moduli of samples before and after each FTC were investigated using a frequency sweep over a range of angular frequency (0.6283 −

62.83 rad/s) at the constant strain within the linear viscoelastic range. G' is a measure of the deformation energy stored in the sample during the shear process, and is related to the elastic behavior of a sample. In contrary, G" is a measure of the deformation energy used up in the sample during the shear and lost to the sample afterwards, representing the viscous behavior of a sample (Tabilo-Munizaga & Barbosa-Cánovas, 2005).

3.2.3.4 Scanning Electron Microscopy

SEM images were performed using the method proposed by Braga & Cunha (2004). Samples (10 mm × 3 mm × 3 mm) of gels were fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) overnight. After rinsing two times in cacodylate buffer, samples were postfixed in 1% buffered osmium tetroxide for 2.5 h. Fixed samples were rinsed again two times with cacodylate buffer and then dehydrated in a graded ethanol series (30, 50, 70 and 90%). Dehydration was continued in 100% ethanol (three changes over 30 min) and overnight dehydration in 100% ethanol. In order to remove the ethanol from the samples, critical point drying (Critical Point Dryer SAMDRI®-PVT-3D, Tousimis Research Co., Rockville, MD, USA) was applied. The dried samples were mounted on aluminum stubs and coated with platinum in a High Resolution Sputter Coater (208HR Cressington High Resolution Sputter Coater with MTM-20 Cressington High Resolution Thickness Controller and Rotary-Planetary-Tilting stage, Cressington Scientific Instruments Ltd., Watford, England). After coating, samples were examined by SEM (Quanta 200F, FEI Company (Field Emision Instruments), Hillsboro, OR, USA) using accelerating voltage of 10 kV under low vacuum conditions.

3.2.3.5 Image Analysis

In order to determine the effect of three different polysaccharide concentration on the pore size of gels, SEM pictures were subjected to ImageJ software Version 1.47a (National Institute of Health, Bethesda, MD, USA). Images were provided as 8bit pictures which is an appropriate format for ImageJ. Then SEM pictures were converted to black (internal part of the pores) and white (walls around the pores) using the threshold command. The grayscale ranged 0-255 and the scale between75-110 were selected to obtain images with cell wall visible. The magnification scale set for all images was 500 μm. For measuring the pores size, each pore selected by a selection tool in software. The number of pores in 500 μm^2 of gel network was also reported.

3.2.3.6 FTIR

FTIR spectroscopy was used to investigate the functional groups of the biopolymers and the conformational changes happening through interactions between the protein and the polysaccharides. FTIR spectra were recorded with FTIR spectrophotometer Nicolet 380 FTIR spectrophotometer (Thermo Electron Inc., San Jose, CA, USA). The analysis of the spectra were conducted using a Nicolet 380 FTIR spectrophotometer (Thermo Electron Inc., San Jose, CA, USA), and spectra were taken between 4000 and 400 cm^{-1} using an attenuated total reflectance (ATR) cell. Twenty spectra were collected for each treatment (N=2; 10 spectra for each), and the mean value of spectra were used for FTIR results presentation. A thin layer of samples were spread on a glass slide and then air-dried under laminar flow at 20°C for 60 min. Finally samples were directly placed onto the diamond crystal cell for spectral measurement.

3.2.3.7 Statistical Analysis

Syneresis measurement were done in triplicate, and mean values and standard deviations reported. The data were subjected to one-way analysis of variance (ANOVA) using SPSS statistical software, (IBM SPSS Statistics 21). Duncan test was performed to determine the statistical differences at 5% significance level. For SEM, three images were taken for each treatment, the size and the number of pores were calculated by ImageJ software (Version 1.47a), and reported as mean ± SD. For FTIR, spectral analysis were conducted using OMNIC software (Thermo Electron Inc., San Jose, CA). The steps involved in the spectra processing included automatic baseline correction, normalization, and smoothing of raw spectra.

3.3 Results & Discussion

3.3.1 Syneresis Measurements

Syneresis, which is the expulsion of water or other liquid from gel, is an important parameter to the stability of the system. Table 3.1 shows the percentage of the syneresis in different samples after 6 h thawing (before centrifugation) and after centrifugation. As can be seen in Table 3.1, the percentage of water separation or syneresis in all samples was increased with an increasing number of freeze-thaw cycles. After the first freeze-thaw cycle, the sample with the ratio of WPI: XC (5:0) had the highest amount of syneresis (20.45%) among all samples. Addition of XCHC significantly reduced the amount of syneresis in the samples. XCHC with the total polysaccharide concentration of 0.25% and 0.5% was capable of decreasing syneresis to 1.36% and zero, respectively. The amount of syneresis in the sample with WPI: XC ratio of (5:0.5) was zero up to 4FTCs and 2.5% after the fifth FTC.

Table 3.1. Syneresis of samples with different ratios of WPI: XC before and after centrifugation over 5 freeze-thaw cycles (FTCs).

	Syneresis (%)					
FTC	WPI: XC (5:0)		WPI: XC (5:0.25)		WPI: XC (5:0.5)	
	Before Centrifugation	After Centrifugation	Before Centrifugation	After Centrifugation	Before Centrifugation	After Centrifugation
1	20.45±0.74	3.33±0.3	1.36±0.05	3.03±0.2	0	1.16±0.1
2	29.54±1	0	11.36±0.5	0	0	1.11±0.08
3	38.64±1.5	0.4±0.02	27.27±1.2	0.15±0.07	0	0.4±0.05
4	28.63±1.3	0.15±0.02	25±1.02	0.08±0.09	0	0.47±0.02
5	45±2.1	0	31.82±1.31	0	2.5±0.12	2.94±0.2

Data were expressed as mean ± SD (n=3)

According to data shown in Table 3.1, the sample with WPI: XC (5:0.5) had the highest water holding capacity. In this sample, water was expelled from the gel after applying external force (i.e, centrifuge), indicating a significant improvement in the freeze-thaw stability of the system.

Figure 3.1 illustrates the total syneresis (before and after centrifugation) of samples over 5 FTCs. As can be seen in this figure the amount of syneresis in all samples increased with each repeated freeze-thaw cycle. The sample without XCHC had the highest amount of syneresis after the fifth cycle (45%). Adding XCHC with total gum concentration of 0.5% significantly reduced the amount of syneresis to 5.44%.

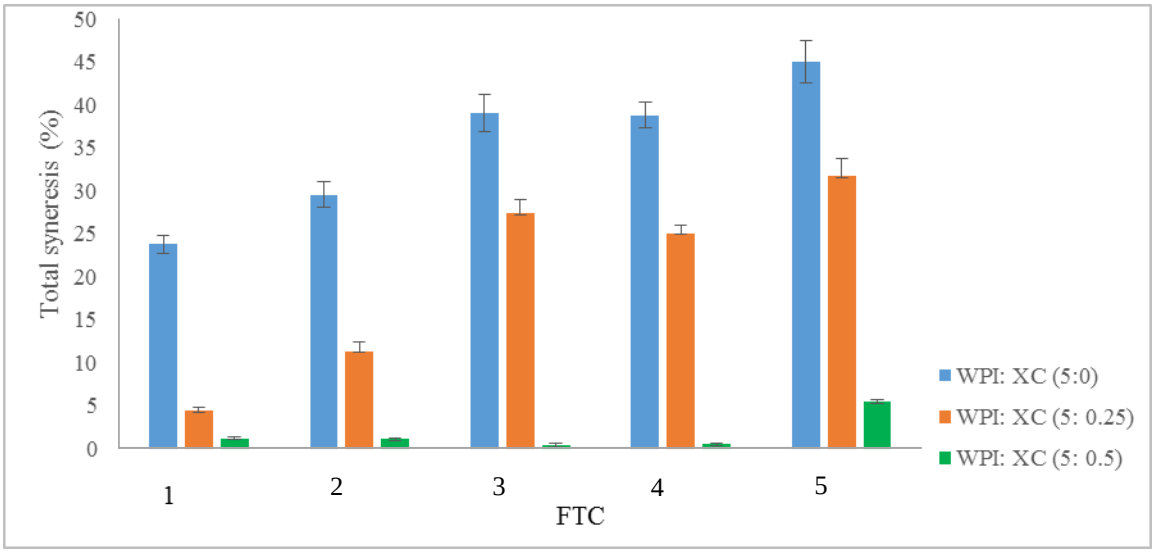


Figure 3.1. Total syneresis (%) of samples with different ratios of WPI: XC over 5 FTCs (n=3).

The syneresis phenomenon during gel storage may be related to the formation of additional interprotein bonds, which decreases the number of active groups for binding water and reduces the intermolecular space for binding water through capillary forces (Zayas, 1997). Xanthan and curdlan hydrogel was able to uniformly distribute into the protein network. The protein network forms a continuous phase that can accommodate the polysaccharide chain, which behaves as a filler of the protein network and improves water holding capacity of the system.

Whey proteins are usually compatible with a large number of polysaccharides, but some incompatibility of WPI and neutral polysaccharide, such as dextran, maltodextrin, and methylcellulose was reported (Turgeon & Beaulieu, 2001). The ability of XCHC to decrease syneresis and hold moisture in the mixtures of WPI, xanthan, and curdlan is dependent on the conformation of curdlan and xanthan in the system. Curdlan is a linear polysaccharide that forms complex tertiary structures which are associated with intramolecular and intermolecular hydrogen bonding (Lo et

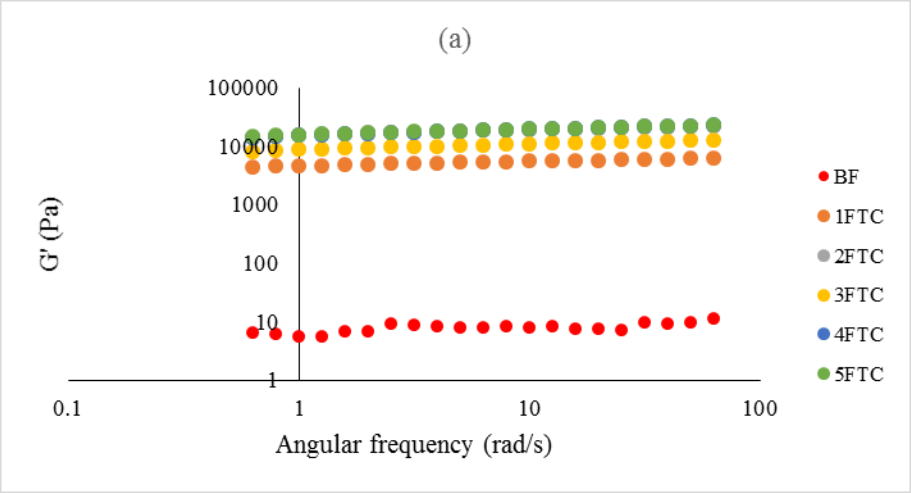
al. 2003). Heating (90°C) of the mixture of xanthan-curdlan solution to form the hydrogel complex could be responsible for the strong initial water-holding capacity (Williams et al., 2009). Xanthan gum is an anionic linear hydrocolloid with a (1→4) linked β-D glucose backbone, and has a large side unit on every other glucose unit at location C-3 (Sworn 2000; Giannouli & Morris 2003). The highly substituted nature of xanthan allows for excellent hydration and hydrogen-bonding activity (Argin-Soysal et al., 2009), which could be considered the main reason why syneresis is reduced when XCHC is added to WPI. In conclusion, the addition of XCHC had a positive effect on the formation of WPI gels.

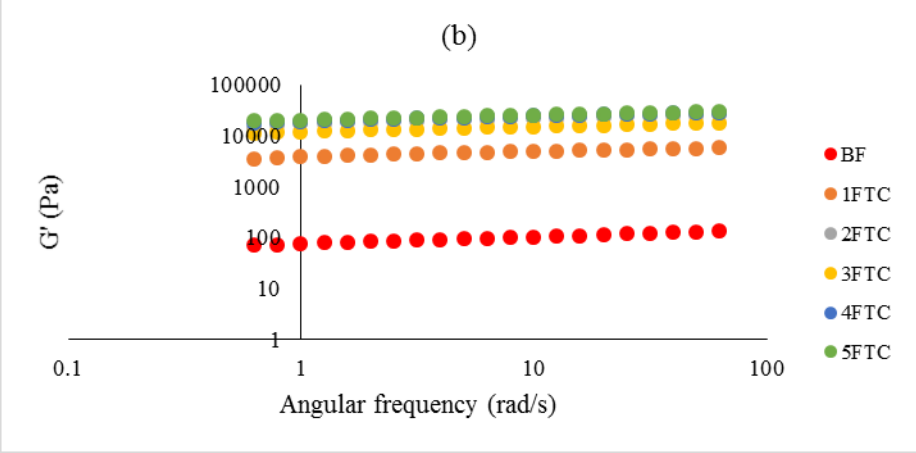
3.3.2 Dynamic Rheological Measurements

In all samples, the elastic behavior dominates the viscous one ($G' > G''$) across the entire frequency range and no crossover point was observed (data not shown). Sample with the WPI: XC ratio of (5:0) was in liquid form prior to being exposed to freezing temperatures, however, after the first freeze-thaw cycle, the liquid changed into a gel network, which explains the significant change in the storage and loss moduli. In samples with WPI: XC of (5:0.25) and (5:0.5) a weak gel network was formed prior to freezing. Therefore, XCHC reduced the minimum concentration of whey protein isolate required to form a gel. A previous study on the rheological behavior of mixed whey protein and galactomannan at pH 7, also showed that the galactomannan had a general positive effect on the WPI gel formation (Tavares & Silva, 2003).

According to data obtained by frequency sweep test, which is shown in Figure 3.2, G' (at the constant frequency of 1 Hz) is 5.66, 75.98, and 409.4 (Pa) for the

samples with WPI: XC ratio of (5:0), (5:0.25), and (5:0.5), respectively. Generally, the amount of G' increased after each freeze-thaw cycle. As can be seen in Figure 3.2, just after the first freeze-thaw cycle, G' increased by 2.5, 1.5, and 1 decade(s) in sample with the ratio of WPI: XC (5:0), (5:0.25), and (5:0.5), respectively. As frequency increased, G' increased within the entire frequency range. After the second freeze-thaw cycle no significant changes of moduli were observed, and sample with WPI: XC (5:0.5) had the least changes of G' from the second to the fifth FTC.





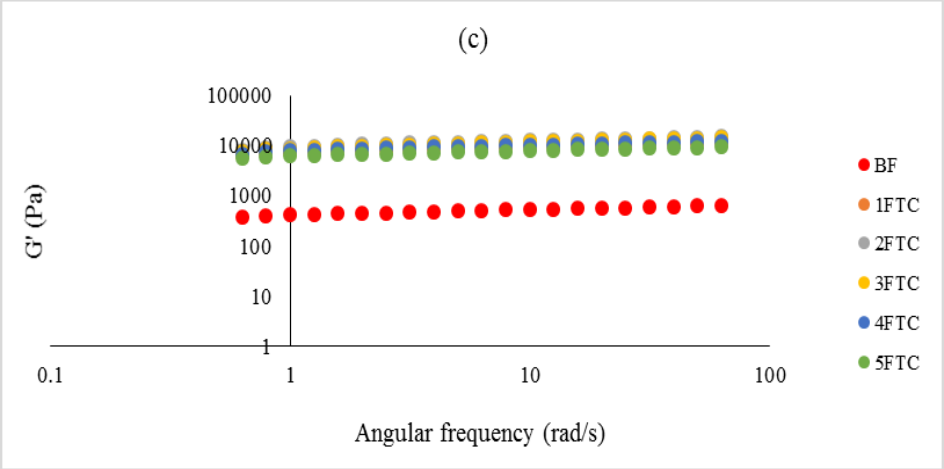


Figure 3.2. Changes of G' vs. angular frequency before freezing (BF) and over five freeze-thaw cycles. WPI: XC of (5:0) (a), (5:0.25) (b), (5:0.5) (c)

3.3.3 Visual Observation of Heat-Induced Gels after 5 FTC

The visual observation of the gels after 5 FTCs is shown in Figure 3.3. The appearance of gels were clear, homogeneous and self-supported (maintained the form when removed from the plastic tubes). This can be attributed to the fact that at neutral pH (≈7), electrostatic repulsions between protein molecules is high, therefore aggregation is hindered and whey protein forms a transparent fine-stranded gel structure. In contrast, when the electrostatic repulsion is low (close to the isoelectric point), protein aggregation happens prior to gel formation, and as a result opaque gels with a particulate microstructure is formed (Langton & Hermansson, 1992; Turgeon & Beaulieu, 2001). Heat-induced WPI gel (Figure 3.3 a) shrank during repeated freeze-thaw cycles and had the maximum amount of syneresis among all samples. On the contrary, mixed gel of WPI and XCHC (Figure 3.3 b, c), showed structural integrity even after the fifth FTC, indicating excellent water retention ability, which could effectively improve texture and freeze-thaw stability of the samples. The

surface area of the gels were measured using ImageJ software and the results are presented in Table 3.2. As can be seen, pure WPI gel with the ratio of WPI: XC (5:0), had the lowest surface area, indicating more shrinkage of the gel after the fifth FTC, which is in agreement with the results obtained from the syneresis measurements of the samples. It should be noted that no significant differences (P>0.05) were observed between the gels with the WPI: XC of (5:0.25) and (5:0.5).

Table 3.2. Surface area of the samples after 5 FTCs determined by imageJ software

WPI: XC	Area (cm^2)
5:0	5.54 ± 0.28^{b}
5:0.25	6.25 ± 0.33^{a}
5:0.5	6.46 ± 0.31^{a}

Different letters within a column indicate significant differences at P< 0.05

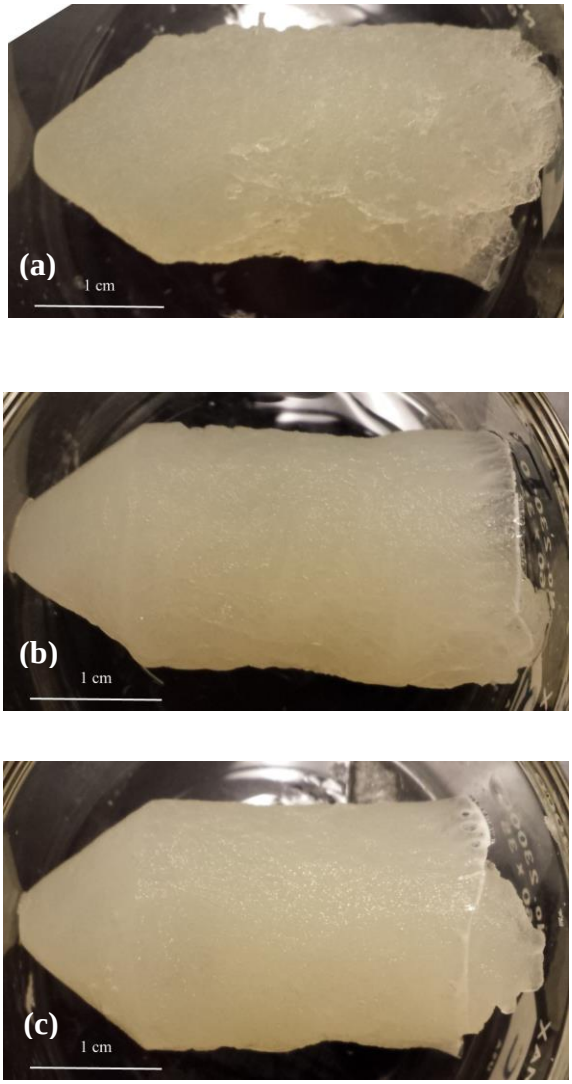


Figure 3.3. Visual observation after the fifth freeze-thaw cycle; WPI: XC of (5:0) (a), (5:0.25) (b), (5:0.5) (c)

3.3.4 SEM and Image Analysis of Heat-Induced Gels after 5 FTCs

SEM and image analysis were used to examine the morphology, microstructure, and pore size of the heat-induced gel networks after 5 FTCs. Figure 3.4 shows original SEM micrographs and corresponding threshold images of the heat induced gels after 5 FTC. As seen in the scanning electron micrographs, addition of XCHC to WPI increased the porosity and decreased the pore size of the gel. It is

possible to see that the structure of the gel was more homogenous with smaller pores at WPI: XC (5:0.5) (Figure 3.4c). In Figure (3.4b), where the WPI: XC is (5:0.25), some network chains is broken after 5 FTCs, whereas at WPI: XC of (5:0.5), no broken chain was observed in the network, indicating strengthening of microstructure and entanglements, and as a result a lower moisture loss over multiple freeze thaw cycles (Figure 3.1). In pure WPI gel (Figure 3.4a), bigger pores are distributed along the gel network.

Original image

Threshold image

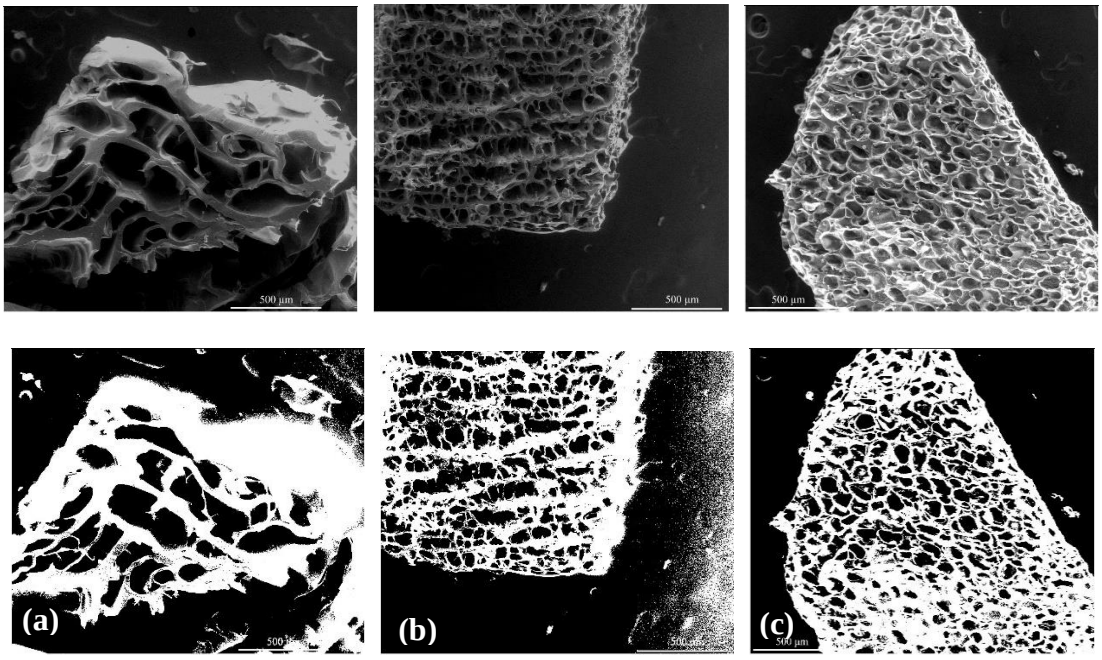


Figure 3.4. SEM micrographs (top row) and corresponding threshold images (bottom row) of the heat induced gels after the fifth freeze-thaw cycle; WPI: XC of (5:0) (a), (5:0.25) (b), and (5:0.5)

The average pore size and the number of pores in a specific area of the gel network (500 μm^2) obtained by ImageJ software is shown in table 3.3. Addition of XCHC at total polysaccharide concentration of 0.25 and 0.5% could increase the porosity of heat-induced whey protein gel to a significant level. As seen in table 3.3

the number of pores increased from 4 (in WPI pure gel), to 24 and 38 in gels with WPI: XC (5:0.25) and (5:0.5), respectively. Furthermore, the pore size in sample with WPI: XC (5:0.5) was 5300 µm2, while in pure WPI gel was 31000 μm^2. Results showed that XCHC can behave as a pore-forming agent in the structure of heat-induced WPI gel, and consequently increases the gel's water retention over multiple freeze-thaw cycles. These are in agreement with syneresis measurements results in gels after 5 FTCs, which showed the improvement of water holding capacity of WPI gels containing XCHC.

Table 3.3. Parameters related to the morphology of the gel networks with three different ratios of WPI: XC after 5 FTC.

Parameters	WPI: XC		
	(5:0)	(5:0.25)	(5:0.5)
*Mean pore size (μm^2)	31000 ± 2200^a	9820 ± 1800^b	5300 ± 1350^c
Number of pores in 500 (μm^2)	4 ± 1^c	24 ± 3^b	38 ± 6^a

*The results are presented as Mean± SD (n=3)

Different letters within a column indicate significant differences at $P< 0.05$

3.3.5. Fourier Transform Infrared (FTIR) Spectroscopy

3.3.5.1. FTIR Spectra of Xanthan, Curdlan, and XCHC

Figure 3.5 shows the FTIR spectra of xanthan and curdlan individually and in combination (XCHC). For pure xanthan, absorptions were observed between 2500-3300 cm^{-1}, which corresponds to the O-H stretching of carboxylic acids formed during the decomposition of ß-D-mannopyranosyl groups present in the side chains of

xanthan. The presence of carboxylic acid can be confirmed by the absorption at 1712 cm^{-1} due to C=O stretching (Lii et al., 2002; Soares et al., 2005; Movasaghi et al., 2008). In addition, C=O stretching was observed at 1600 cm^{-1}. The FTIR results for pure xanthan showed medium absorptions at 1380 cm^{-1}, 1252 cm^{-1}, 1151 cm^{-1}, and 1028 cm^{-1} which are related to COO^{-}, CH, C=O for glucose units, and C-O respectively (Movasaghi et al., 2008).

For pure curdlan, several bands were observed between 1200 and 850 cm^{-1} in the spectrum, the same region where the C-O stretching modes are found. The assignment for absorptions at 3290 and 2860 cm^{-1} are associated with the OH stretching, and peaks at 1650, 1150, and 1370 cm^{-1}, are respectively related to C=O, C-O stretching, and CH (Jin et al., 2006; Gao et al., 2008, Gagnon & Lafleur, 2007, 2011). The weak band at ~890 cm^{-1} could be assigned to the ß linked glucosidic bonds (Gagnon & Lafleur, 2007). For XCHC, the FTIR spectrum was closer to the curdlan spectrum than xanthan, indicating that curdlan contributes more than xanthan in the peaks associated with the functional groups of XCHC. Addition of xanthan to curdlan enhanced the intensity of the peaks in curdlan.

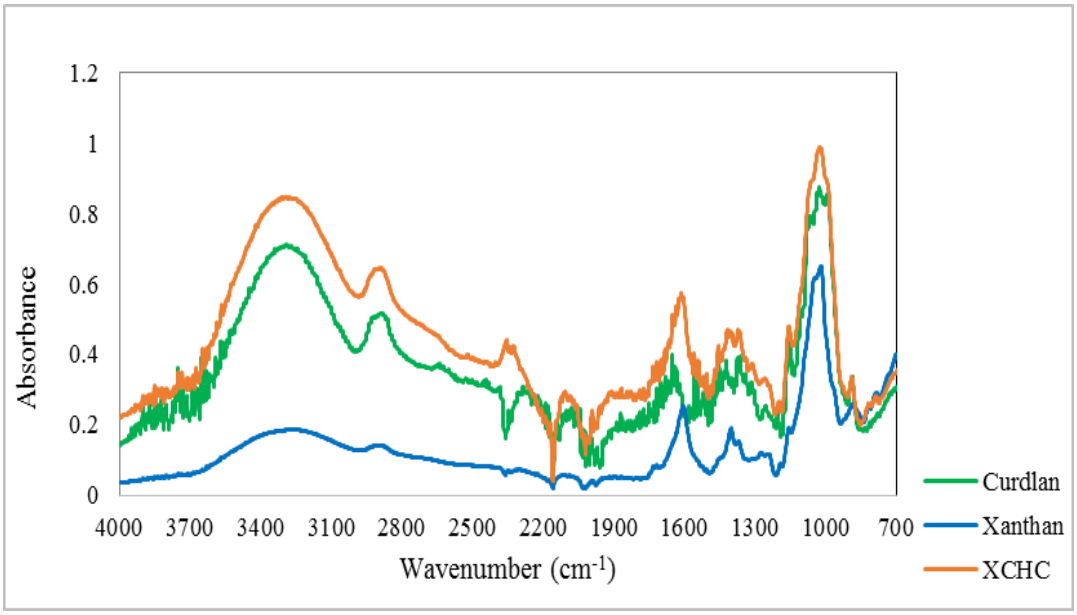


Figure 3.5. FTIR spectra of xanthan, curdlan, and XCHC (1% w/v)

3.3.5.2. FTIR Spectra of WPI

FTIR spectra of whey protein isolate is shown in Figure 3.6. Two regions of interest were observed in the WPI spectra: 3600-3000 and 1700-1400 (amide I bond and Amide II bond), these results were in agreements with previous studies (Le Tien et al., 2000; Jiang et al., 2010). Two peaks at 3070 and 3270 cm^{-1} were observed in the region between 3000 to 3600 cm^{-1}, which can be associated with OH and NH stretching mode. Other researchers also reported the same assignment for the WPI and heated WPI. They mentioned that peaks in the FTIR spectra of WPI are not influenced by thermal treatments, however, irradiation, and mixing of WPI with other biopolymers might affect the peaks (Le Tien et al., 2000; Jiang et al., 2010).

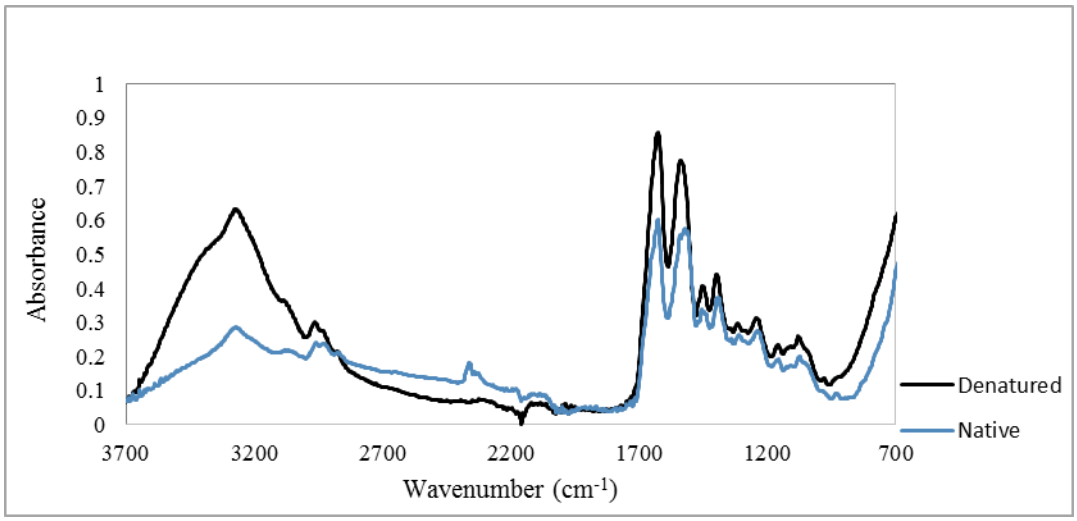


Figure 3.6. FTIR spectra of native and heat denatured WPI (10% w/v)

The region between 1700-1600 cm^{-1} is related to Amide I absorption, which is sensitive to the secondary structure of the protein, and primarily represents the stretching vibrations of the C=O bond of the amide group (Jiang et al., 2010). In this region, several peaks (1692, 1682, 1668, 1661, 1652, 1645, 1635, 1626 and 1618 cm^{-1}) were observed. The bands at 1661, 1652, and 1645 cm^{-1} could be attributed to α-helix/unordered segments, and the band at 1635 cm^{-1} was associated with the amide groups involved in the extended β-sheet structure, which are related to α-lactalbumin and *ß*-lactoglobulin in whey protein structure. The peak at 1668 could be assigned to the turns (Movasaghi et al., 2008; Le Tien et al., 2000; Jiang et al., 2010). These bands were rarely affected by the aggregation process (Jiang et al., 2010). The peaks at 1682 and 1622 cm^{-1} were related to the formation of intermolecular antiparallel β-sheets (Jiang et al., 2010).

Comparison of the FTIR spectra of unheated and heated WPI showed that heating treatment (90ºC for 30 min), increased the intensity of the bands at 1617 cm^{-1}

and 1682 cm^{-1}. These bands are assigned to the intermolecularly hydrogen-bonded structures (Ismail et al., 1992). Similar results were also reported by Geara (1999). They reported that at pH 7, these two bands which are associated with the aggregation of the protein will be appeared at the temperatures above 80°C. Generally, heating WPI increased the intensity of the peaks, which is in agreement with other researchers' results (Morr & Ha, 1993; Fang & Dalgleish, 1998; Geara, 1999; Lee et al., 2007). Increasing the peak intensity shows the protein denaturation, aggregation and gelation (Morr & Ha, 1993; Lee et al., 2007).

The structure of the WPI and its responses to the heat treatments depend on the pH and the concentration of whey protein (Geara, 1999; Lee et al., 2007; Dissanayake, 2011). It has been reported that, unheated WPI has more discriminated bonds at lower pH values such as 3, compared to higher pH values (≈7) (Geara, 1999). In this study, the pH of the WPI was 7, and no big differences were observed between native and heated WPI.

3.3.5.3. FTIR Spectra of Mixed WPI and XCHC

The FTIR spectra of the heat-induced gels are presented in Figure 3.7. The band corresponding to amide I (1600-1700 cm^{-1}) depends on the secondary structure of the protein and it is the most commonly used band for the quantitative analysis of the secondary structures. Moreover, absorption associated with the amide II bond (1400-1500 cm^{-}) leads mainly to bending vibrations of the N-H bond. Therefore, changes of these bands might clarify how polysaccharide addition can influence the secondary structure of the protein.

Addition of XCHC to the WPI, increased the frequencies of the peaks assigned to the amide I and II regions. However, the band assigned to the carboxylate group in the polysaccharides at 1600 cm^{-1} was not noticeable in the mixed WPI-XCHC, indicating the interactions between the WPI and XCHC and the fact that carbohydrate molecules have lower capacity to form intermolecular hydrogen bonds between themselves in the presence of whey protein (Guerrero et al., 2014). In addition, the intensity of Amide I peaks in mixed WPI-XCHC was lower compared to the pure WPI gel, indicating conformational changes occurring through interactions between protein and the polysaccharides. However, the bonds at 1680 and 1690 cm^{-1}, which are associated with the β-sheet and turns, were stronger in the mixed WPI-XCHC gel compared to the pure WPI gel. This might be due to the formation of intermolecular antiparallel β-sheets. Extended antiparallel β-sheets are commonly found in aggregated proteins, especially in heat-denatured proteins (Guerrero et al., 2014).

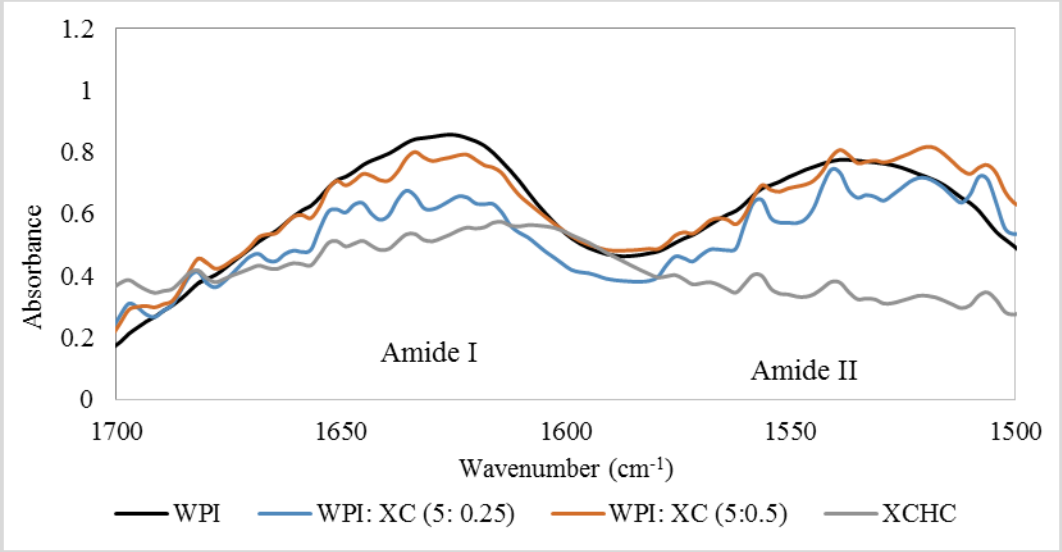


Figure 3.7. FTIR spectra of heated WPI, XCHC, and mixed gels

These results imply that aggregation was occurred and these aggregates were partly composed of β-sheets. This observation represents that regular structures and unordered segments converted to intermolecular β-sheets. The β-sheet structure has a higher degree of intermolecular hydrogen bonding and explains the important role of the water molecules during the unfolding–folding process. The FTIR spectra and the properties of protein-polysaccharide mixtures depends on the polysaccharide origin. For examples, an increase has been reported in the mixture of carboxymethylcellulose and soy protein isolate at 1680 cm^{-1}, however, when dextran and gum Arabic were mixed with soy protein isolate, the bond decreased (Guerrero et al., 2014).

3.4 Conclusion

Over the five FTCs investigated, addition of xanthan-curdlan hydrogel complex showed excellent ability to improve freeze-thaw stability and water holding capacity of the heat-induced whey protein gels. Adding XCHC at a total polysaccharide concentration of 0.5%, could eliminate syneresis up to four freeze-thaw cycles, and increased the storage modulus of the gel by seventy times compared to pure WPI gel, prior to freezing. Therefore, XCHC reduced the minimum concentration of whey protein isolate required to form a gel. Furthermore, it minimized the discrepancies of G' in frequency sweep test over 5 FTCs, indicating improvement of the stability of the system. XCHC, as a pore-forming agent, could increase the porosity and decrease the pore size in the heat-induced WPI gels. The smaller the size of the pores, the more uniformly they can be distributed in the structure, and therefore the water holding capacity of the system was increased over multiple freeze-thaw cycles. The FTIR spectrum of XCHC was closer to the curdlan

spectrum than that of xanthan, indicating curdlan provides more OH functional groups than xanthan in the mixture. Curdlan had an additive effect in the intensity of the functional groups of XCHC. Addition of XCHC to whey protein heat-denatured gel was able to convert regular structures and unordered segments to form more intermolecular β-sheets, which have a higher degree of intermolecular hydrogen bonding.

Mixed gel with WPI: XC ratio of (5:0.5) showed excellent structural integrity even after five freeze-thaw cycles without any broken chain in the gel network, demonstrating excellent water retention ability and freeze-thaw stability of the samples. The ability of XCHC to strengthen the rheological properties of heat-induced WPI gel and to eliminate syneresis suggests that it could explore the possibility of new applications in novel food formulation, in particular in the dairy-based frozen food products.

Chapter 4: The Effects of pH on Rheology of Whey Protein Isolate and Xanthan-Curdlan Hydrogel Mixed Gels

4.1 Introduction

The interest for studying the behavior of mixed gels is increasing due to the improvement of mechanical and structural properties of mixed gels compared to that of pure gels. Food products are complex systems of hydrocolloids, in which texture is mainly controlled by the presence of proteins and polysaccharides. Studies of the interactions of hydrocolloids in aqueous solutions and gels are crucial to developing novel formulated foods. Furthermore, controlling desirable functional properties of food systems is not achievable without using hydrocolloids. Mixed gels, obtained from solutions of biopolymers, are either complex (intermolecular attraction) or incompatible (intermolecular repulsion). The macromolecular distribution in solution results in antagonist or synergistic effects on the formation of mixed gels (Bertrand & Turgeon, 2007). Improvement in the gelling properties of mixed gels have been reported previously (Ould Eleya & Turgeon, 2000; Shim & Mulvaney, 2001; Wang & Qvist, 2000; Bertrand &Turgeon, 2007).

Gels formed by several gelling agents can be divided into three groups: interpenetrating, coupled, and phase-separated networks (Morris, 1986). Interpenetrating networks are formed when two macromolecules form gel separately, so the networks are independent. Both networks spread the entire system, but any interaction between them is mostly topological (Morris, 1986; Zasypkin et al., 1997). Coupled or complex coacervate networks are formed in the presence of favorable

intermolecular interactions between the different types of polymers. In contrast, when polymers are incompatible and interactions between them are repulsive (or when their affinity towards the solvent is different), phase-separated gels are formed (Tolstoguzov, 1991, 1995). Structural and rheological properties of mixed gel are influenced by internal and external factors such as pH, ionic strength, temperature, polymer concentration, ratio of protein to polysaccharide, the charge of the proteins and polysaccharides (Tulstoguzov, 1991). In mixed systems containing protein and anionic polysaccharide, pH affects both protein self-association and protein–polysaccharide cross-association. Complex coacervation is observed at pH values, where the two polymers carry opposite charges (below the isoelectric point of the protein). At pH values above the isoelectric point of the protein, biopolymers carry similar negative net charges; thus, complex formation is hindered, and thermodynamic incompatibility is supported (Ould Eleya & Turgeon, 2000).

Whey proteins (WP) are widely used as a food ingredient due to their nutritional and useful functional properties. Gelling is one of the most important functional properties of WP, which is the result of an aggregation process induced by changing the conditions, usually by increasing the temperature. Gelling properties of WP depend on several factors, such as pH, salt concentration, temperature, heating rate, and ionic strength (Zayas, 1997; Stading & Hermansson, 1990). When the electro-static repulsions are strong, gels from globular proteins are transparent with a fine-stranded structure, whereas in conditions of weak electrostatic repulsions, they are opaque with a coarse, lumpy, and particulate structure (Verheul & Roefs, 1998).

As shown in a previous study that a hydrogel complex (XCHC) formed by xanthan, a negatively-charged hydrocolloid, and curdlan gum, a neutral, linear microbial polysaccharide, could significantly reduce syneresis over five freeze-thaw cycles, while retaining rheological and textural properties (Williams et al., 2009). Furthermore, addition of XCHC to WP isolate (WPI) solution, showed excellent ability to form a homogeneous and well-structured heat-induced gel, with a significant lower syneresis compared to pure WPI gel over multiple freeze-thaw cycles (Chapter 3).

The aim of this study was to investigate the effects of pH on the rheology of a mixture of WPI and XCHC upon heating and cooling, and to examine the influence of XCHC on the gel transition temperature of WPI gel.

4.2 Materials & Methods

4.2.1 Material

Odorless, fine, free-flowing white powder curdlan containing a minimum of 90% β-D-glucan and with a maximum of 10% water was used (Takeda Vitamin & Food USA, Orangeburg, NY). Xanthan (TICAXAN®) was kindly supplied by TIC Gums (Belcamp, MD); WPI (Hilmar 9400) by Hilmar ingredients (Hilmar, CA). The WPI used in this study consisted of 93.4% protein (% dry basis), Lactose (0.2%), fat (0.6%), moisture (4%), and ash (2.6%).

4.2.2 Preparation of Solutions

4.2.2.1 Xanthan-Curdlan Hydrogel Complex

To prepare xanthan curdlan hydrogel complex (XCHC, 1% w/v) an equal amount of each of the biopolymer powders was weighed and dry blended at ambient temperature and gradually poured to deionized water under constant stirring. Gum solutions were agitated for 15 minutes to achieve a homogeneous, lump free aqueous solution. The biopolymer solution was magnetically stirred while gradually being heated on a heat plate until the temperature of the solution reached 90°C. After cooling to room temperature, the pH of the solution was adjusted to 4, 5, 6, and 7 using 0.1 N of HCL or NaOH. Biopolymer solutions were covered and refrigerated overnight at 4°C to allow complete hydration.

4.2.2.2 Whey Protein Isolate Solution

WP stock solutions 20% (w/v) were prepared by slowly dissolving the dried powder in deionized water agitated with a magnetic stirrer for 2 hrs at room temperature to ensure complete dissolution. Solutions were then stored overnight at 4°C for complete hydration. The pH of WPI solutions was adjusted to 4, 5, 6, and 7 using 1 N of HCL or NaOH.

4.2.2.3 Mixtures of WPI and XCHC

Mixtures containing XCHC (0.5%) and WPI (10%) were prepared by mixing equal amounts of the stock solutions of WPI and XCHC at desired pH values. The solutions were stored overnight at 4ºC prior to the rheological measurements. Pure WPI and XCHC with the same concentration of 10% and 0.5% were prepared to study the behavior of individual biopolymers.

4.2.3 Methods

4.2.3.1 Dynamic Oscillatory Measurements

Dynamic rheological measurements were performed at a frequency of 1 Hz at a constant strain within the linear viscoelastic region (LVR), using an AR2000 Rheometer (TA Instruments, New Castle DE) with a standard-size DIN geometry. Each sample was poured directly into the measuring system of the rheometer cup at the desired temperature, and left standing for 2 minutes to allow structure recovery and temperature equilibration. The dehydration of the samples was limited by using a solvent trap. In amplitude sweep tests, the strain was increased from 0·01 to 100% at a constant frequency of 1 Hz to obtain the linear viscoelastic region (LVR). Temperature sweep tests were done at a frequency of 1 Hz within the strain of LVR. Solutions were heated from 40 to 90ºC, held at 90ºC for 30 min, cooled to 20ºC, and then kept for 15 min at 20ºC. Finally, samples were heated to 70ºC and held for 15 min. The temperature increment of 2ºC/min was applied for both heating and cooling ramps. At pH 4, WPI and XCHC formed insoluble complexes, which were poured into the geometry.

4.3 Results & Discussion

4.3.1 Effect of pH on XCHC

Figure 4.1 shows the storage modulus (G') of XCHC (0.5%) during the temperature treatments for four different pH values from 4 to 7. As seen in Figure 4.1, the changes of G' during the temperature ramps are almost identical for pH values of 5, 6, and 7. As temperature increased from 40 to 90°C and held at 90°C for 30 min,

the storage modulus of XCHC reduced. Upon cooling from 90 to 20°C, G' started to increase and reached close to its initial G' at 40°C (≈1Pa). This can be attributed to the resistance of XCHC structure over the wide range of temperature (Williams et al., 2009), which prevents the structure of the solution from being collapsed at 90°C and holding for 30 min at this temperature. During the holding time at 20°C, G' reached a plateau, and finally reduced during the reheating (from 20 to 70°C) and holding time at 70°C. Results show that G' values varied slightly with decreasing pH from 7 to 5; thus, the rheological properties of XCHC were not pH-dependent, and no significant differences were observed between the storage modulus of hydrogel systems within the pH range of 5 to 7. However, G' of the XCHC was significantly lower at pH 4, indicating less elastic or solid behavior of hydrogel at this pH. Changes of G' during heating and cooling of the hydrogel at pH 4 was similar to that at other pH values. According to the changes of storage modulus as a function of temperature or time, gelation did not occur in XCHC during the applied thermal treatments. Xanthan is one of the non-gelling polysaccharides, which is widely used in food products as a thickener or stabilizer and has been reported to form weak gels at concentrations of 0.5%. Moreover, curdlan is one of the heat-gelling polysaccharide, which can form either low-set or high-set gels. Results from temperature sweep tests revealed that XCHC with total polysaccharide concentration of 0.5% did not form gel upon heating and cooling in the applied thermal treatments.

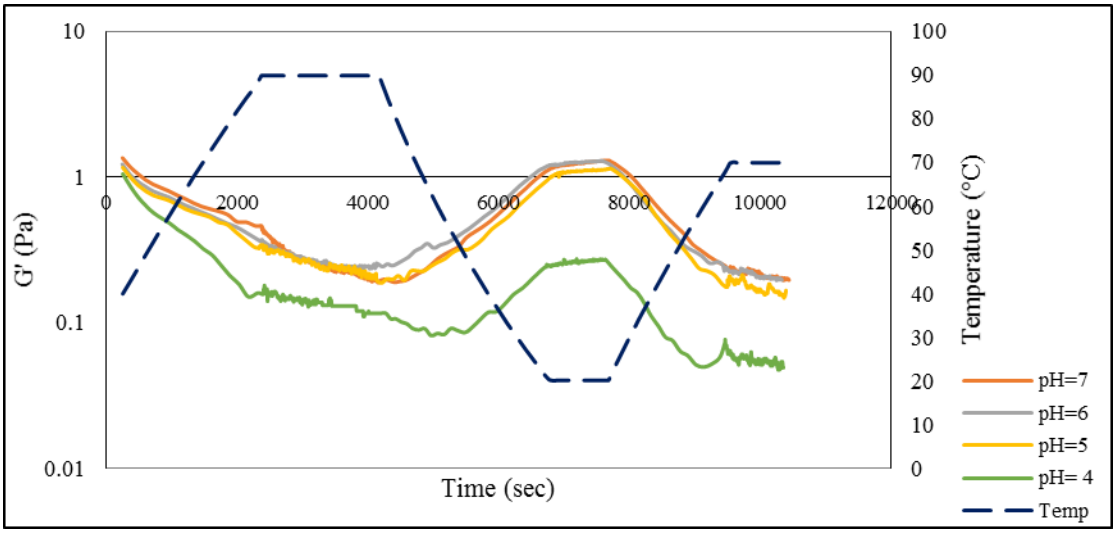


Figure 4.1. Time–temperature dependence of G′ for XCHC (0.5%) over the pH range of 4-7 during heating and cooling.

4.3.2 Effect of pH on Gelation of WPI

The effect of pH on gel formation and melting of WPI solution (10%) within pH range of 4–7 is shown in Figure 4.2. During the first heating ramp from 40 to 90°C, G' did not change significantly up to the point that a steep rise in G' was observed. This point can be considered as the onset of WPI gelation. The temperature at which G' started to increase sharply is defined as gelation temperature. As can be seen in Figure 4.2, gelation temperature is pH dependent in WPI. The onset of gelation occurred at about 79°C for pH values of 4 and 5, and 85°C for pH 6. By contrast, at pH 7, G' did not change notably during the first heating treatment; therefore, WPI did not form gel upon first heating from 40 to 90°C. G' started to increase erratically after the holding time at 90°C, but not as sharply as the G' in other pH values. During the 30 min holding time at 90°C, G' of the whey protein gels was found to increase. Upon cooling from 90 to 20°C, the storage modulus of gels increased monotonically. During the 15-minute holding time at 20°C, G' reached a

plateau and then decreased as temperature increased from 20 to 70°C. During the holding time of 70°C, G' reached a second plateau. Previous studies also reported an increase in the magnitude of the gel modulus with decreasing temperature for whey protein gels (Cooney et al., 1993; Manoj et al., 1997; Ould Eleya & Turgeon, 2000); this was attributed to the reinforcement of attractive forces (hydrogen bonding, van der Waals forces) between the protein particles in the gel (Manoj et al., 1997). The gel formed at pH 6 showed the highest G' among all gels. The second highest storage modulus was observed in the gel formed at pH 4, followed by gel formed at pH 5. The weakest gel with the lowest amount of G' was seen in the gel formed at pH 7. These results are in agreement with previous studies on the changing of gelation temperature of WPI at different pH values, which showed that the onset of gelation of *β*-Lactoglobulin occurred at lower temperatures in the acidic pH range compared to the neutral pH range (Stading & Hermansson, 1990; Ould Eleya & Turgeon, 2000). They also reported that the highest G' of *β*-Lactoglobulin gel was observed at pH 5.5, which supports the results obtained in this study, with highest G' seen at pH 6.

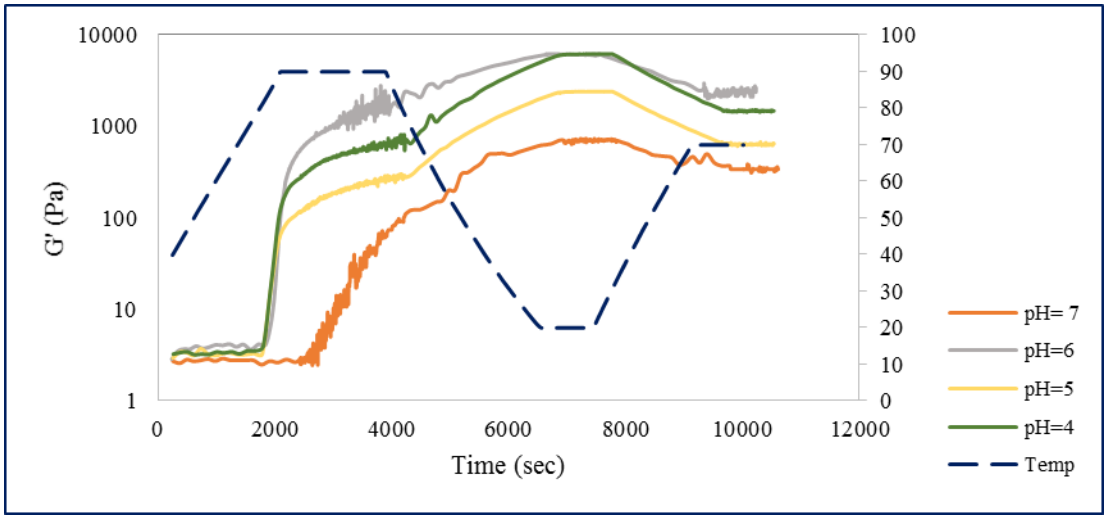


Figure 4.2. Time–temperature dependence of G′ for WPI (10%) over the pH range of 4-7 during heating and cooling.

Figure 4.3 displays the visual observation of WPI gels at different pH values formed in the rheometer cup after performing thermal treatment. Results from other studies on the microstructure and physiochemical properties of gels formed from thermally denatured whey proteins support the visual appearance observed in this study. When the electrostatic repulsion between protein molecules is strong, fine-stranded networks are formed (Figure 4.3c,d). Whey proteins only have a limited number of hydrophobic patches on their surface, so interactions at other regions on the protein surface are hampered in the presence of strong electrostatic repulsion. On the other hand, near the isoelectric point of protein, where the net charge is relatively low (Figure 4.3a,b), gels have a particulate network. The microstructure of whey protein gels affects the optical properties as well; fine-stranded gels are transparent, and particulate gels are opaque. This is due to the low scattering efficiency of thin protein strands, and strong scattering efficiency of the protein particles (Foegeding et

al., 1995; Doi, 1993; Langton & Hermansson, 1992; Chantrapornchai & Mc Clements, 2002).

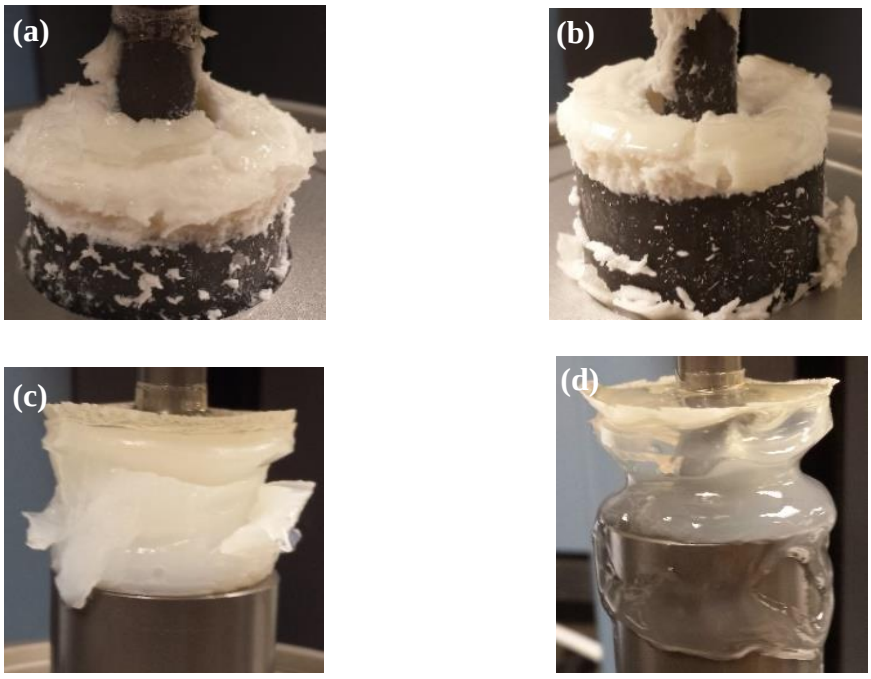


Figure 4.3. Visual observation of WPI gels (10%) formed in the rheometer cup after performing thermal treatment; pH 4 (a), 5 (b), 6 (c), and 7 (d)

4.3.3 Effect of pH on Gelation of Mixed Gels

The effect of pH on gel formation and melting of the mixture of WPI and XCHC is shown in Figure 4.4. As can be seen, gelation temperature and the storage modulus of the gels were strongly pH-dependent. At all pH values except for pH 4, G' decreased as temperature increased from 40°C, and reached its minimum level at 76°C, 79°C, and 82°C for pH 5, 6, and 7, respectively, after which drastic increase of G' was observed. During the holding period at 90°C, G' continued to increase. However, at pH 4, G' was almost constant up to 76°C, then increased remarkably up

to 85°C. Upon cooling from 90 to 20°C, G' in all pH values increased monotonically. During the holding period at 20°C, G' reached a plateau, followed by a slight decrease upon reheating to 70°C. Samples reached a second plateau during the holding period at 70°C, except at pH 7, in which G' increased during the holding time at 70°C.

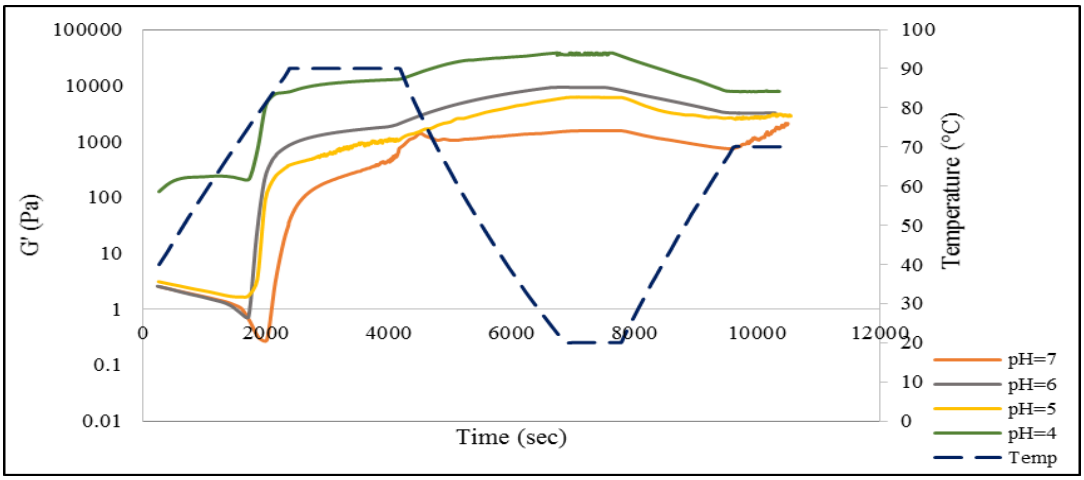


Figure 4.4. Time–temperature dependence of G′ for mixed WPI (10%) and XCHC (0.5%) over the pH range of 4-7 during heating and cooling.

The trends of G' changes as a function of time or temperature, in mixed gels, are very similar to those in WPI gels, except for the first step of the temperature sweep test (heating from 40 to 90°C). During this thermal treatment, G' of WPI samples was constant at the beginning and then increased, while in mixed WPI-XCHC samples, G' initially decreased and then increased. The reason that G' decreased at the beginning of heating was probably because of the heating effect on the XCHC structure, which decreased the storage modulus of the hydrogel (Figure 4.1). Furthermore, an increase in G' was due to the whey protein network

consolidation and consequently WPI gel formation. Prior to consolidation of WPI network, the effect of XCHC dominated the effect of WPI in mixed systems, however, after formation of WPI network whey protein controlled the rheological behavior of the system. The different behavior of mixed gel at pH 4, compared to other pH values, can be attributed to the formation of protein-polysaccharide complexes, due to the electrostatic attraction of biopolymers at this pH. As seen in Figure 4.4, mixed gel at pH 4 had a significantly higher storage modulus than that of mixed gels over pH 5-7. In contrast, gel formed at pH 7 had the least G' compared to other pH values. Results showed that pH had a strong effect on the rheological behavior of WPI and XCHC mixed gels. According to Figure 4.4, mixed gels showed two distinct behaviors over the pH range of 4-7: over pH 5–7, and at pH 4.

Visual observation of WPI-XCHC mixed gels at different pH values formed in the rheometer cup after performing thermal treatment is shown in Figure 4.5. Over pH 5–7, biopolymers are incompatible (they carry a similar negative charge), and phase-separated gels are formed. At pH 5 and 6, around the isoelectric point of WPI, self-association of whey protein is enhanced, and as a result, segregation of protein–polysaccharide is increased. In this situation, thermodynamic incompatibility increases, which inhibits the cross-association of protein and polysaccharide (Figure 4.5b,c) (Tolstoguzov, 1991). In contrast, at pH 7 (far from the isoelectric point of whey protein), the protein self-association is reduced, but due to the strong electrostatic repulsive forces, cross-association of WPI and XCHC is also reduced (Figure 4.5d). At pH 4, electrostatic complexes between WPI and XCHC are formed prior to protein gelation, which may explain the different behavior observed for the

mixed gel at this pH. Therefore, at pH 4, WPI and XCHC associated, and then formed a coupled or complex coacervate network (Figure 4.5a). Previous studies also reported the formation of complex networks for oppositely charged polymer mixtures, such as gum Arabic and gelatin (Peters et al., 1992).

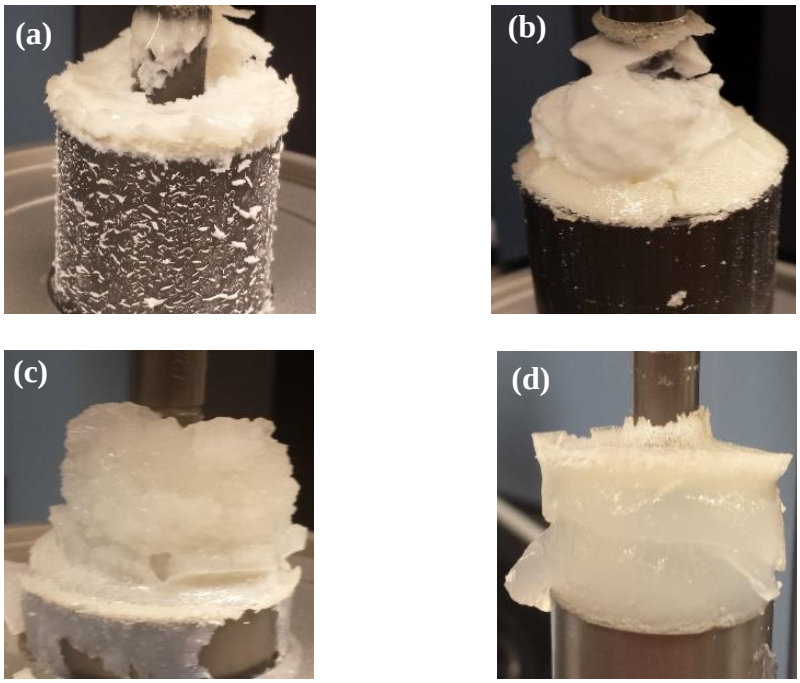


Figure 4.5. Visual observation of mixed WPI (10%) and XCHC (0.5%) gels formed in the rheometer cup after performing thermal treatment; pH 4 (a), 5 (b), 6 (c), and 7 (d)

4.4 Conclusion

Gelation temperature and the storage modulus of pure WPI gels were strongly pH-dependent. However, WPI gels formed at different pH values showed similar rheological behavior. In the pH range of 4-6, WPI gels were formed upon first heating from 40 to 90°C, next reinforced by cooling to 20°C, and then weakened during reheating from 20 to 70°C. The onset of gelation temperature of WPI was lower in acidic pH ranges compared to the neutral pH; 79°C for pH values of 4 and 5, and

85°C for pH 6. At pH 7, WPI did not form gel upon first heating from 40 to 90°C, and G' started to increase erratically during the holding time at 90°C. In mixed gels, the presence of XCHC increased storage modulus of the gels. Rheological behavior was pH-dependent and almost similar to that of the pure WPI gel: at the beginning XCHC controlled the rheological behavior of the mixture, and after the formation of WPI network, the behavior was led by the whey protein isolate. Over pH range of 5-7, gels were phase-separated, while at pH 4 complex coacervate network formed. Comparison of the storage modulus as a function of temperature or time in pure WPI and mixed WPI-XCHC, supports the synergistic effects of these biopolymers on increasing the elastic modulus of the gels after the consolidation of WPI network.

Mixed protein polysaccharide gels can exhibit improved functionality over protein and polysaccharide gels alone. Manipulating these macromolecular interactions by controlling pH can assists food scientists in gaining new insights into the rheology and microstructure of novel mixed systems in the food industry. Useful knowledge in this area can benefit food manufacturers to create unique and fabricated formulations with superior sensory and physiochemical properties that are more acceptable to consumers.

Chapter 5: Characterization of the Interactions between Whey Protein Isolate and Xanthan-Curdlan Hydrogel Complex in Aqueous Solutions

5.1 Introduction

Proteins and polysaccharides are present together in many kinds of food systems, and both these hydrocolloids have an important role in texture, structure, stability, and rheological characteristics of food. These biopolymers are extensively used because they are natural polymers, and compared to various synthetic materials, are biocompatible, biodegradable, and non-toxic. Whey proteins (WP) are widely used in the food industry for their well-known high nutritional and functional characteristics. Formation of complexes of WP with polysaccharides is one of the useful techniques to manipulate and improve functional properties of WP. Protein-polysaccharide interactions find many applications in new food formulation, and food scientists can develop novel foods with new functional properties by controlling the interactions of these macromolecules. To date, many researches have studied the interactions of proteins and polysaccharides in aqueous solutions (Sanchez et al., 1997; Tolstoguzov, 1997; Dickenson, 2003; Jones et al., 2010; de Kruif et al., 2004; Benichou et al., 2007; Bertand and Turgeon, 2007; Doublier et al., 2000; Turgeon et al., 2003; Weinbreck et al., 2004; Ibanoglu 2002; Sanchez and Renard, 2002). However, despite the broad improvements made in recent years, polysaccharide and protein interactions in food hydrocolloids continue to challenge researchers.

Functional properties of biopolymers can be modified by the presence of other types of biopolymers. For example, solubility, conformational stability, gel forming

ability, surface activity, emulsifying properties, and foaming properties of many proteins are greatly influenced by their interactions with polysaccharides. Benichou et al. (2007) reported that addition of xanthan gum successfully enhanced the surface hydrophobicity of WP, and as a result improved its emulsifying properties. Klein et al. (2010) found that the mixture of whey protein isolate and gum Arabic was able to stabilize oil in water emulsions better than each of the biopolymers alone. Fibrous complexes formed from WPI–xanthan gum were used to manufacture meat analogues or other textured products (Chen & Soucie, 1985; Baer et al., 1992). Laneuville et al. (2000) created particulated and irregular complexes from WPI-microfluidized xanthan, with an appropriate structure applicable as a fat substitute.

The compatibility, stability, and characteristics of protein-polysaccharide complexes depend on different factors, such as pH, ionic strength, ratio of protein to polysaccharide, charge, total concentration of biopolymers, quality of solvent, molecular weight, salt and sugar, and the temperature of the medium (Samant et al., 1993; Glahn & Rolin, 1995; Weinbreck et al., 2004; de Kruif & Tuinier, 2001). Interactions between protein and polysaccharide may be associative or segregative. Two oppositely charged polymers in an aqueous solution can form complex coacervation (associative phase separation) due to their electrostatic attraction (for instance complexation between proteins and anionic polysaccharides below the isoelectric point of protein). This leads to the formation of a two-phase system that consists of a mixed biopolymer complex phase suspended in a solvent phase depleted in both biopolymers. Depending on the electrical characteristics of the biopolymers and the composition of the solution, this complex may be either soluble or insoluble.

Segragative phase separation (thermodynamic incompatibility) occurs due to the net repulsion between biopolymers at pH above the isoelectric point of protein, where they carry the same charge (Dickinson, 1998; Tolstoguzov, 1997; Schmitt et al., 1998).

Combination of xanthan gum, an anionic microbial polysaccharide with a disaccharide backbone and a trisaccharide side chain, and curdlan, a neutral microbial linear polysaccharide consisting of D-glucose with β-1, 3 glucosidic linkages, showed excellent ability to eliminate syneresis over multiple freeze-thaw cycles (FTCs) (Williams et al., 2009). Furthermore, the addition of xanthan-curdlan hydrogel complex (XCHC) could eliminate or reduce syneresis in heat-induced whey protein gel after repeated freeze-thaw cycles (Chapter 3). Studying the interactions between whey protein and xanthan-curdlan hydrogel can provide food scientist new opportunities to create novel formulations in the application of proteins and polysaccharides, such as microencapsulation, edible films and coatings, and production of meat analogous and fat substitutes in frozen products by improving their freeze-thaw stability. The main goal of this study was to determine the effect of pH and Pr: Ps ratio on interactions between whey protein isolate and xanthan curdlan hydrogel complex (WPI –XCHC). To achieve this aim, several analytical techniques such as zeta-potential, turbidity, and particle size were employed.

5.2 Materials & Methods

5.2.1 Materials

Odorless, fine, free-flowing white powder curdlan containing a minimum of 90% β-D-glucan and with a maximum of 10% water was used (Takeda Vitamin &

Food USA, Orangeburg, NY). Xanthan (TICAXAN®) was kindly supplied by TIC Gums (Belcamp, MD); WP isolate (WPI) (Hilmar 9400) by Hilmar ingredients (Hilmar, CA). The WPI used in this study consisted of 93.4% protein (% dry basis), Lactose (0.2%), fat (0.6%), moisture (4%), and ash (2.6%).

5.2.2 Preparation of Solutions

5.2.2.1 Xanthan-Curdlan Hydrogel Complex

To prepare XCHC equal amounts of each of the biopolymer powders were weighed and dry-blended at ambient temperature and gradually poured to the stock phosphate buffer solution (pH=7, 5mM) under constant stirring. Gum solutions were agitated for 15 minutes to achieve homogeneous, lump free aqueous solutions. The biopolymer solutions were magnetically stirred while gradually being heated on a heat plate until the temperature of the solution reached 90°C. After cooling to room temperature, the biopolymer solutions were covered and refrigerated overnight at 4°C to allow complete hydration. The total concentration of gums (xanthan and curdlan) in the hydrogel solution was 1% (w/v).

5.2.2.2 Whey Protein Isolate Solution

WP stock solution (1% (w/v)) was prepared by slowly dissolving the dried powder in stock phosphate buffer solution (pH=7), agitated with a magnetic stirrer for 2 hrs at room temperature to ensure complete dissolution followed by overnight storage at refrigerator (4°C) for a complete hydration.

5.2.2.3 Mixtures of WPI and XCHC

Biopolymer mixtures containing WPI (0.1%) and XCHC (0, 0.02, 0.05 and 0.1%) were prepared by mixing different ratios of the stock solutions with buffer

solutions at the desired pH values (2-8). The pH of the mixtures was adjusted to the appropriate value before mixing. Mixtures were stored overnight at 4°C before analysis.

5.2.3 Zeta Potential Measurements

The electrical charge of individual biopolymers and biopolymer mixtures were measured by a laser Doppler Velocimetry (Zetasizer Nano ZS90, Malvern, U.K.). Zeta potential were determined through measurement of 1 mL of samples in disposable folded capillary cell (DTS1070, Malvern Instrument, Inc.) with 12 measurements per sample. Zeta potential was obtained by converting the measured electrophoretic mobility using the Smoluchowski Theory.

5.2.4. Particle Size Measurements

Hydrodynamic diameters of different samples were measured by a dynamic light scattering (DLS) instrument BI-200SM Goniometer Version 2 (Brookhaven Instrument Corp., Holtsville, New York, USA) equipped with a 35 mW He-Ne laser beam at a wavelength of 637 nm. The actual laser power was 10 Mw. All measurements were performed at 25°C.

5.2.5 Turbidity Measurements

The amount of soluble protein present in the aqueous phase was determined by measuring the absorbance of transparent solutions at 280 nm. Turbidity measurements were carried out with a UV–visible spectrophotometer (DU-730 UV/VIS spectrophotometer, Beckman Coulter Inc., Fullerton, CA, USA) at a wavelength of 280 nm. The supernatant of samples after centrifugation (1000 rpm for

10 min) was placed in a 10mm path-length cuvette, and the turbidity (τ) was then measured as a function of time at 25°C. The turbidity was defined as $\tau = -\ln(I/I_0)$. In this formula, I is the light intensity that passes through a volume of solution in a 1 cm cube, and I_0 is the incident light intensity.

5.2.6 Statistical Analysis

Zeta potential, turbidity, and particle size measurement were done in triplicate, and mean values and standard deviations reported. The data were subjected to one-way analysis of variance using SPSS statistical software, (IBM SPSS Statistics 21). Duncan test was performed to determine the significant differences at the 5% probability level.

5.3 Results & Discussion

5.3.1 Zeta Potential Measurements

Electrical forces are generally the major driving force for the interaction of charged biopolymers (Harnisilawat et al., 2006). Both proteins and ionic polysaccharides have charge densities, which are related to their composition in amino acid and uronic acid and pyruvate groups, and the charges depend on the pH and ionic strength (de Kruif et al., 2004; Weinbreck et al., 2003). Zeta potential of WPI solution and XCHC at different pH values from 2 to 8 is shown in Figure 5.1. The zeta potential of WPI (0.1% wt %) went from +15.8 to -25.03 as the pH increased from 2 to 8. Similar results were obtained for zeta potential of β-Lactoglobulin (0.1%) (Jones et al., 2010). The pI of WP according to Figure 5.1 is around 4.5, which is very close to the isoelectric point of WPI that has been reported

previously (≈pH 4.6) (Ennis & Mulvihill, 2000). Zeta potential of XCHC (0.1% wt %) went from -8.85 to -40.83 as the pH increased from 2 to 8. Curdlan is a neutral polysaccharide and xanthan is an anionic polysaccharide, so the negative charge of XCHC is due to the negative charge of xanthan gum. The anionic property of xanthan stems from the presence of uronic acid component and the carboxylic groups that are directly bound to the pyranose ring. Xanthan has carboxyl groups on the side chains, so the negative zeta potential decreases as pH decreases. The zeta potential for xanthan gum (0.03 wt %) was reported from -70 to -90 within the pH range of 4 to 6 (Le & Turgeon, 2013). As seen in Figure 5.1, a noticeable decrease in the magnitude of the negative charge on the XCHC was observed, when the pH decreased below 4. This can be associated with the partially protonation of anionic carboxylic ($–COO^-$ to –COOH) in the pH range of 2-4, which is below the PKa of the carboxylic acid (Harnisilawat et al., 2006).

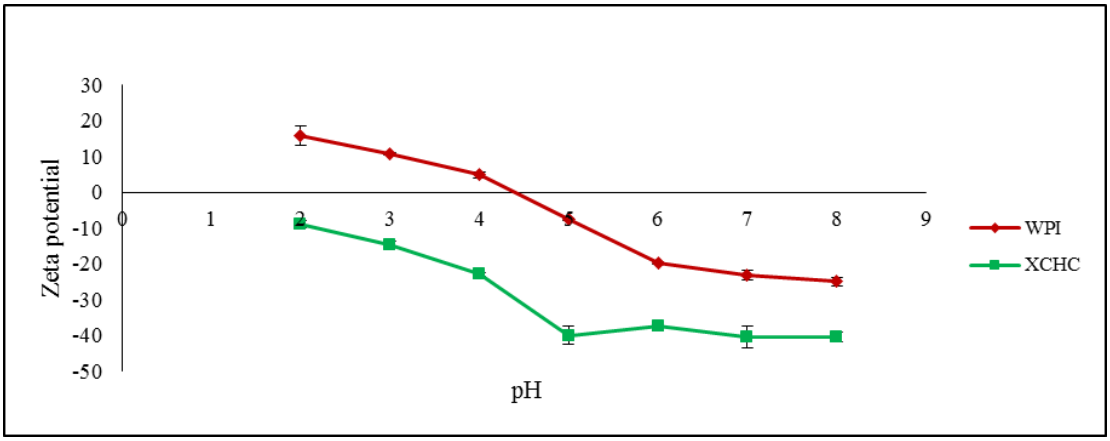


Figure 5.1. Zeta potential of WPI and XCHC (0.1% w/v) as a function of pH.

The zeta potential of WPI, XCHC, and mixtures with different ratios of biopolymers at a pH range of 2 to 8 is shown in Figure 5.2. It should be noted that over pH range of 2-4, due to the electrostatic attractive forces, there were large insoluble complexes in the mixed biopolymer solutions that were too big to place in the measurement cell, so the zeta potential of the supernatant of the mixtures was measured and reported. The zeta potential of the protein-polysaccharide mixture with ratio of WPI: XC (0.1:0.1) went from +14.03 to -35.63 as pH increased from 2-8. The magnitude of the zeta potential in mixtures of WPI and XCHC is somewhere between protein and polysaccharide alone, indicating the positive charge from the whey protein dominated the negative charge from the polysaccharide. At pH 4.5, the net charge on WPI is close to zero because this pH is near to the electrostatic point of the whey protein. When the pH decreased from 5 to 2, the zeta potentials of mixed solutions were more positive than that of the pure xanthan gum solution, indicating that the electrostatic complexes had been formed. Therefore, pH 5 can be considered as the start point of protein polysaccharide complex formation.

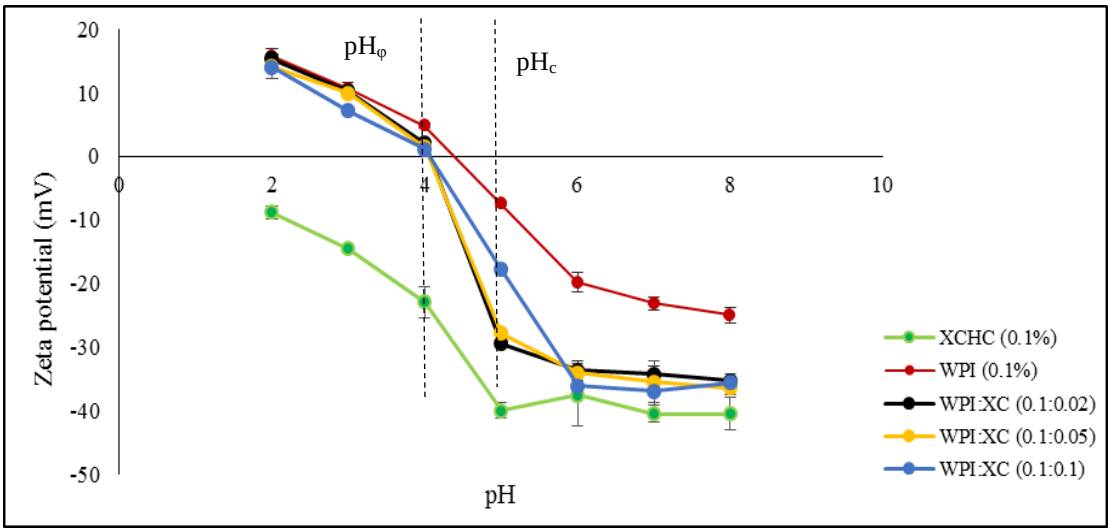


Figure 5.2. Zeta potential of mixed WPI (0.1%) and XCHC (0-0.1%) as a function of pH.

No significant differences were observed in the magnitude of zeta potential in mixtures with different WPI: XC ratio, except at pH 5. At this pH zeta potential of the mixed solution with WPI: XC ratio of (0.1:0.1) was more positive than the other two mixtures, representing higher amounts of electrostatic complex formation at higher concentrations of xanthan and curdlan. At pH 5, whey protein and XCHC initiate to form soluble complexes, and this point can be considered as critical pH (pH_c). Weinbreck et al. (2003, 2004) found similar pH_c for complex formation of WPI - Carrageenan (5.5) and WPI-gum Arabic (5.3). They also mentioned that pH_c in mixed WPI and polysaccharide with larger zeta potential is higher, due to the stronger interactions between biopolymers. At lower pH values (pH=4), where the interaction is stronger, phase separation occurs (pH_φ), and finally the formation of complex is suppressed due to the charge reduction in the XCHC (Winbreck et al., 2003). Soluble complexes can form at pH values above the isoelectric point of WPI, at which the protein and polysaccharide carry the same net charge. This can be attributed to the presence of positive surface charges of protein, which are called "patches". The formation of protein and polysaccharide complex at pH 5 might be due to the attraction between oppositely charged "patches" on the surface of the protein and negatively charged XCHC (Weinbreck et al., 2003). The charge density in these areas is higher than the net protein surface charge density, and a weak electrostatic attraction is expected to occur (Doublier et al., 2000). This phenomenon has been previously reported in mixed systems containing whey protein and a secondary biopolymer, such as pectin, carrageenan, sodium alginate (Dalgleish & Hollocou, 1997; Zaleska et al., 2000; Sanchez et al., 1997; Neiser et al., 1998).

As seen in Figure 5.2, the isoelectric point of mixed WPI-XCHC shifted to a lower point compared to the isoelectric point of WPI alone (from 4.5 to 4 mV), so the mixed system neutralized sooner than the WPI alone, and after that the net charges of the mixed solutions were similar to that of the pure protein, meaning that the electrostatic complexes had been neutralized and the net charge is because of the free proteins in the mixed solutions. At pH 4, the anionic polysaccharide bound to the amphoteric protein molecules and the origin of this binding is electrostatic binding of anionic carboxyl groups on the xanthan gum to cationic amino groups on the whey protein surface.

Visual observation of mixed WPI and XCHC in the pH range of 2-5 is shown in Figure 5.3. The results obtained from zeta potential measurements were in agreements with visual observations. The most concentrated precipitant was observed at pH 4, where the biopolymers carry the most opposite charges and the interactions are stronger. At pH 5 no precipitant and insoluble complexes were observed, indicating the formation of soluble complexes due to the presence of positive charges on the surface of protein. The pH boundaries ($pH_{c,}$ pH_{φ}) were primarily due to the formation of a complex between ß-lactoglobulin and polysaccharide, and it is believed that the contribution of α-lactalbumin would appear at lower pH values (Weinbreck et al., 2004).

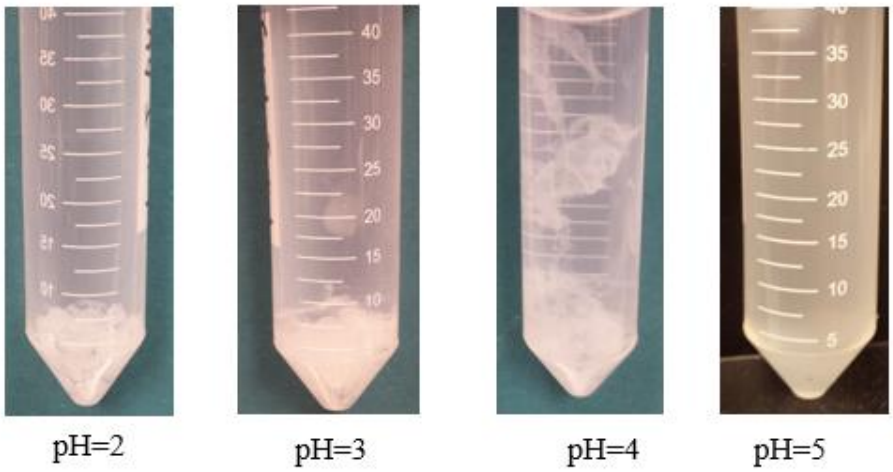


Fig 5. 3. Visual observation of mixed solutions of WPI (0.1%) and XCHC (0.05%).

5.3.2 Particle Size Measurements

Figure 5.4 shows the mean diameter of the particles in WPI and XCHC solution with biopolymer concentration of 0.1%.

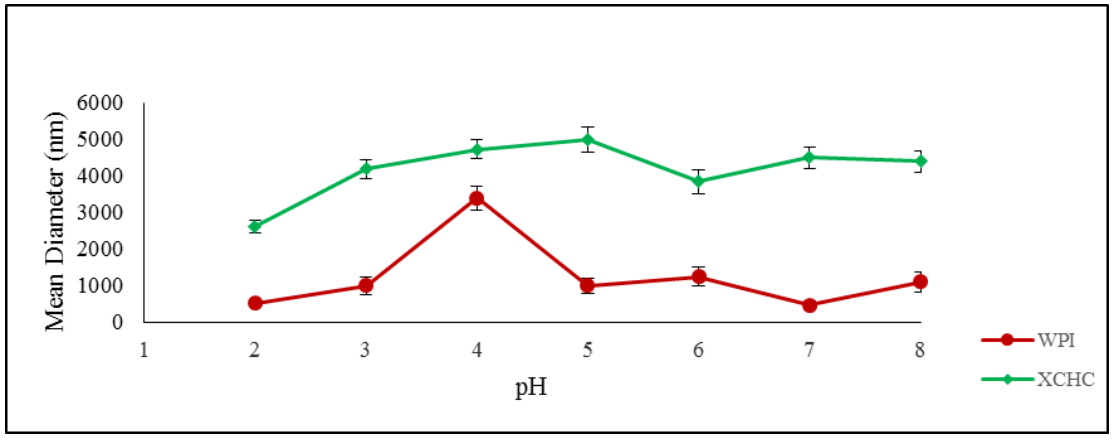


Figure 5.4. Mean diameter of WPI and XCHC (0.1%) as a function of pH.

As seen in Figure 5.4, for WPI the largest particle size was observed at pH 4. The driving force for protein aggregation around the isoelectric point is a combination

of hydrophobic attraction, van der Walls attraction and some electrostatic attraction between positive groups on one protein and negative groups on another (Harnsilawat et al., 2006). The self-association of WPI at pH values around its isoelectric point is confirmed in the literature (Majhi et al., 2006; Syrbe, 1998) and can be attributed to the relatively weak electrostatic repulsion between the protein molecules.

Regarding whey protein, the higher the charge magnitude of the particle the smaller the mean diameter of the particle. At high charge magnitude the electrostatic repulsion is high, preventing protein particles from being close to each other and forming large aggregates. In contrast, for XCHC, the changing trend of particle size versus pH, did not accord with the aforementioned hypothesis; the minimum particle size of XCHC was observed at pH 2, where the magnitude of the charge was at its minimum level, thus the size of the particle were not pH-dependent in XCHC.

For mixed WPI- XCHC, as previously mentioned, insoluble complexes were formed at pH values below the isoelectric point of whey protein (≈4.5) due to an electrostatic attraction between the biopolymers. These complexes in the mixed biopolymer solutions were too big to place in the measurement cell. Therefore for pH values of 2, 3, and 4, the particle size of the supernatant of mixtures were measured and reported. Mean diameter of particles in mixed WPI-XCHC is shown in Figure 5.5.

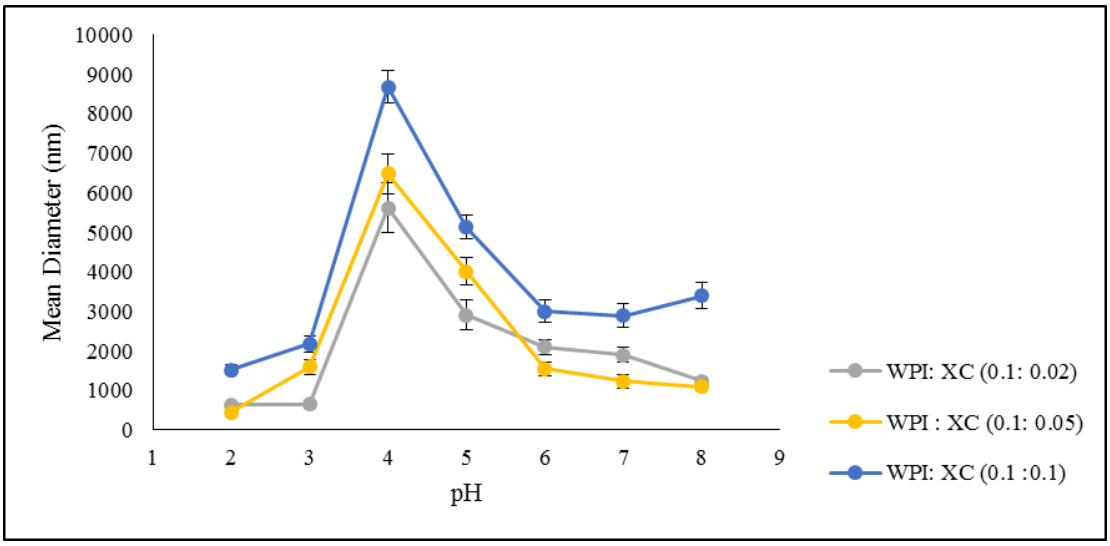


Figure 5.5. Mean diameter of the supernatant of mixed WPI (0.1%) and XCHC (0.02, 0.05, and 0.1%) as a function of pH.

As seen in Figure 5.5 the largest particle size in the supernatant was observed at pH 4. At a pH value close to the isoelectric point, the attraction between proteins and polysaccharides is at its maximum level and large insoluble and soluble aggregates tend to form. Above pH 4.5, where no complexation was formed due to the repulsive attractions, the largest particle size was seen at pH 5. This pH is defined as pH_c (the pH at which the formation of soluble protein-polysaccharide complexes is initiated). Soluble complexes are formed at this pH, due to the presence of positively charges patches at the surface of the whey protein (Weinbreck et al., 2004). As the pH increased from 5, the mean diameter of particles decreased, which can be attributed to the strong electrostatic repulsion between biopolymers molecules at pH values far from the isoelectric point, which prevents the formation of large aggregates.

Generally, increasing the percentage of XCHC in the mixture increased the mean diameter of the particles in the WPI-XCHC mixtures. The rate of protein

recovery through polysaccharide complexation is approximately 90% (Chen & Soucie, 1985; Samant et al., 1993). Therefore there is always some proportion of protein and polysaccharide which remain uncomplexed (Laneuville et al. 2000). As mentioned before, the mean diameter of particles in mixed WPI-XCHC (at pH range 2- 4) represents the particle size of the supernatant of WPI-XCHC, so the higher particle size in the samples containing more XCHC is because of the presence of further uncomplexed polysaccharide, which remained in the supernatant. Moreover, the existence of the polysaccharide in the solution decreases the formation of the large protein-protein aggregates by increasing the viscosity and lowering the rate of colliding particles (Laneuville et al. 2000). This might be the reason of the lower mean diameter of the sample in WPI: XCHC ratio of (0.1:0.05) than the ratio of (0.1:0.02) at pH 6 and 7.

5.3.3 Turbidity Measurements

Figure 5.6 displays the optical density (O.D.) of WPI and mixed WPI-XCHC solutions at 280 nm. The amount of soluble protein was determined by measuring the O.D.sub.280 (Harnsilawat et al., 2006). The results showed that the maximum amount of soluble protein in all samples was observed at pH 5. This data supports zeta potential data and visual observations, which indicated that the formation of soluble WPI-XCHC complexes occurred at $pH_c \approx 5$. Formation of soluble WPI and XCHC complexes is attributed to the interaction between oppositely charged "patches" on the surface of the protein and negatively charged XCHC.

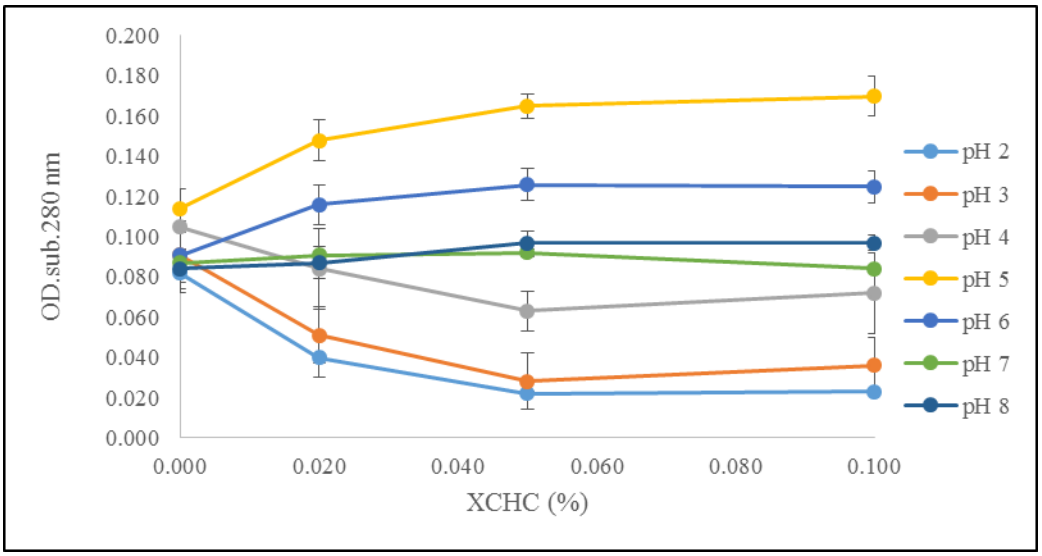


Figure 5.6. Dependence of the OD.sub.280 on XCHC percentage in solutions containing WPI (0.1%) and XCHC (0-0.1%).

At pH 2, 3, and 4 there was a noticeable decrease in free protein with an increasing percentage of XCHC, showing that more protein was incorporated into the insoluble complexes. Harnisilawat et al. (2006), have reported similar results regarding the effect of polysaccharide (sodium alginate) on soluble protein in the mixed WPI-Alginate. At pH 5 and 6, the amount of soluble protein increased as the percentage of XCHC concentration increased. The presence of the polysaccharide in the system limits the formation of protein–protein aggregates by increasing the viscosity of the solution and lowering the rate of the colliding particles. Therefore, more free proteins remain in the supernatant of the mixed WPI-XCHC. At pH 7 and 8, the soluble protein concentration remained almost unchanged and it was independent of XCHC concentration; this might be due to the strong electrostatic repulsion between the biopolymer molecules at pH values far from the protein isoelectric point, and the fact that whey protein was not incorporated into an insoluble

complex. As seen in figure 5.6 increasing the percentage of XCHC from 0.05 to 0.1%, did not have any significant effect on the amount of soluble protein.

In summary, according to the analytical experiments and visual observation of this study, depending on the pH (2-8) of the system, there are three different possible mode of interaction between WPI and XCHC, which is shown schematically in figure 5.7.

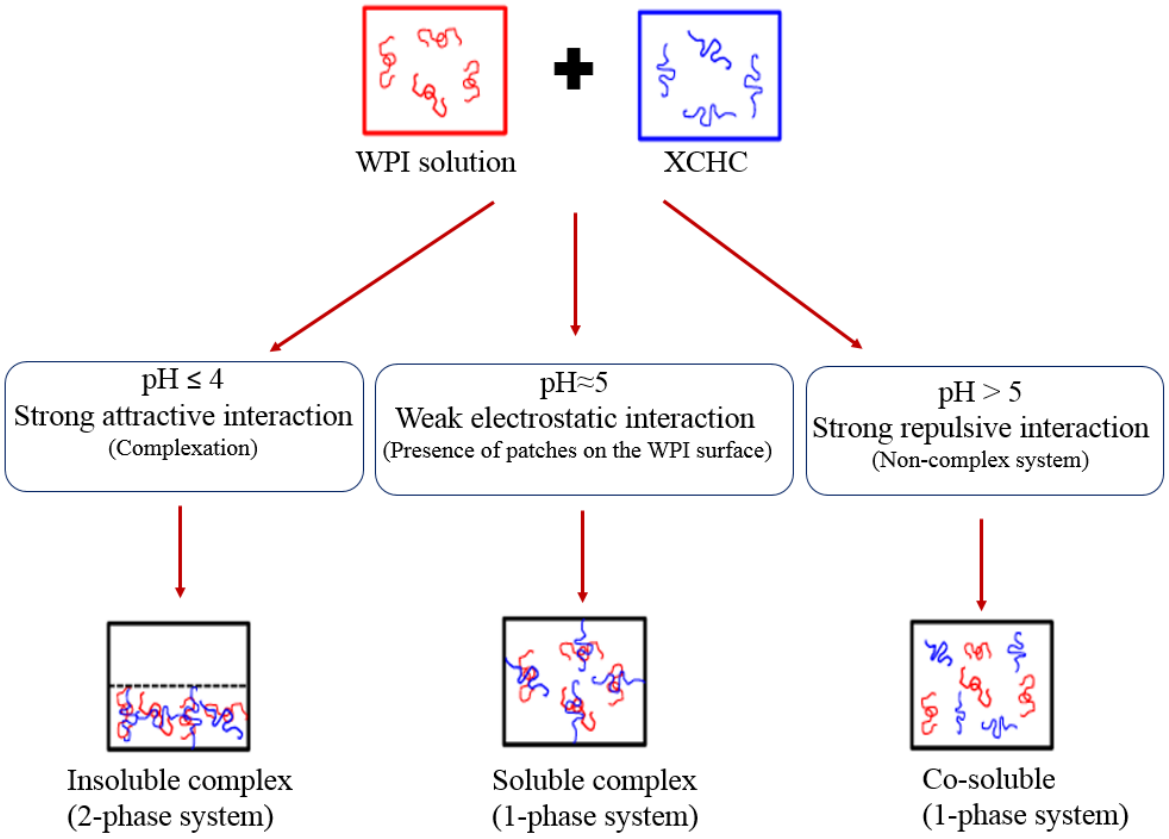


Figure 5.7. Schematic representation of the possible mode of interaction between WPI and XCHC within the pH range of 2-8

5.4 Conclusions

The formation of WPI and XCHC complexes resulted from electrostatic interactions. The zeta potential, particle size, turbidity, and soluble protein measurements provided useful results about the interactions of WP and XCHC in aqueous solutions. Results showed that WPI could interact with xanthan-curdlan hydrogel and form soluble or insoluble complexes depending on the pH. Insoluble complexes were formed at pH values where the protein and polysaccharide had opposite electrical charges (pH 2, 3 and 4) due to the strong electrostatic attractive forces between the biopolymers. Soluble complexes were formed at $pH_c \approx 5$, where XCHC was negatively charged and whey protein was positively charged and had little net charge. Formation of soluble complexes can be attributed to the binding of anionic XCHC to positively charged patches on the surface of the whey protein. At pH values of 6, 7, and 8, no complexation was observed due to strong electrostatic repulsive forces between the biopolymers. Results also showed that in the mixed WPI-XCHC, the isoelectric point is lower compared to WPI, indicating the mixed system neutralized sooner than the WPI alone, and the electrostatic complexes had been neutralized and the net charge is because of the presence of free proteins in the mixed solutions. The knowledge gained from this study regarding the interactions of WPI and XCHC could be applicable to design novel food ingredients with unique functionalities, especially in frozen products for which repeated freezing and thawing is a major concern in the quality loss of the product. The application of WPI-XCHC complexes as an edible film in frozen vegetables, surimi and seafood, or as a meat analogue in frozen vegetarian nuggets, can be interesting areas for further research and applications.

Chapter 6: Efficacy of the Whey Protein Isolate, Xanthan, and Curdlan Mixed as an Edible Coating in Frozen-Thawed Mushroom *(Agaricus bisporus)* and Green Bell Pepper

6.1 Introduction

Edible coatings are defined as a thin layer of material that covers the surface of the food product and can be eaten as part of the whole product. The major components of edible coatings are polysaccharides, proteins, and lipids (Vargas et al., 2008). Formulation of composite coatings is one of the interesting research areas for food scientists, as they can take advantage of the special functional properties of each group and improve their weaknesses (Donhowe & Fennema, 1994). Edible coatings are capable of protecting perishable food products from deterioration by suppressing respiration, decreasing drip loss, improving textural quality, retaining volatile flavor compounds, and reducing microbial growth (Debeaufort et al., 1998). Interest in the use of bio-based coatings and packaging material is increasing due to their ability to prolong shelf life and improve quality of fresh, frozen, and processed food products (Diab et al., 2001).

In fresh and processed meat, poultry, and seafood products, edible coatings can improve the quality of the product by retarding moisture loss, and by reducing lipid oxidation and discoloration (Gennadios et al., 1997). Edible coatings can also be used in processed fruits and vegetables to improve the structural integrity of frozen fruits and vegetables and prevent the moisture absorption and oxidation of freeze-dried fruits or vegetables (Olivas & Barbosa-Vanovas 2005; Park et al., 2005). Furthermore, edible coatings can carry functional ingredients such as antioxidants,

antimicrobials, nutrients, and flavors that improve food stability, quality, functionality, and safety (Lin & Zhao, 2007). The combination of soy protein and a cellulose-based coating could reduce permeability of the coating to oxygen and water vapor, and decrease weight loss in the cut apple and potato (Bladwin et al., 1996). Chitosan coatings in strawberry significantly slowed the ripening of strawberries, reduced the weight loss, and delayed the changes in the color of the product (Hernandez-Munoz et al., 2008). Han et al. (2004, 2005) reported chitosan coatings could prolong the shelf life of fresh strawberries and red raspberries by decreasing weight loss and delaying changes in color, titratable acidity, and pH during cold storage. They also reported that coating was capable of reducing drip loss and improving the texture quality of frozen-thawed strawberries.

Previous studies have reported positive effects from edible coating on the shelf life and quality of mushrooms and bell peppers during storage periods at room or refrigerator temperature. Hershko and Nussinovitch (1998) reported that coated mushrooms with an alginate-based solution showed better appearance and color, and less weight loss by comparison with uncoated mushrooms. Chitosan and chitosan–glucose complex can be used as edible coating in mushrooms for maintaining quality and extending their postharvest life (Eissa, 2007; Jiang et al., 2012). Ochoa-Reyes et al. (2013) studied the effect of different types of polysaccharide when used as an edible coating, on the shelf life quality of green bell peppers. They reported that gum Arabic was able to improve the quality of the product at room temperature over a period of ten days. Coating peppers with hydrocolloid-lipid blends was effective in

maintaining quality during refrigerated storage, and was able to reduce the weight loss of the peppers (Conforti & Zinck, 2002).

Freezing is an effective method of preservation that maintains the quality of foods close to the fresh product. Consumption of frozen vegetables has increased by 20% during the last 20 years (USDA, 2014). During the freezing process undesirable changes in color, flavor, and texture occur, originating mainly from the activation of natural enzymes in the food product (Martínez-Romero et al., 2003). Generally, frozen vegetables are known to be more pleasant, and to maintain a better color, than canned vegetables.

Moisture migration and ice recrystallization are two main physical changes that occur during frozen storage. Both phenomena are related to the stability of a product's frozen water, which affects the texture, weight loss, and nutrient loss upon thawing of the vegetable (Goncalves et al., 2011). One of the useful methods to delay those changes during the freezing process is to apply edible coatings on the frozen products. Several studies on increasing the shelf life and quality of frozen thawed seafood products through coating solutions have been undertaken (Klincceker et al., 2009; Stuchell & Krochta, 1995; Sathivel, 2005; Song et al., 2011; Jeon et al., 2002), but little research has been done on frozen fruit and vegetable using coating procedure. Moreover, the use of milk protein-based coatings, which are odorless and flavorless, can be beneficial for controlling enzymatic browning and delaying loss of quality in frozen vegetables. The objective of this study is to evaluate the effects of coatings made with whey protein isolate (WPI), xanthan, and curdlan on the quality of mushrooms and bell peppers during a ten day frozen storage.

6.2 Materials & Methods

6.2.1 Materials

Odorless, fine, free-flowing white powder curdlan containing a minimum of 90% β-D-glucan and with a maximum of 10% water was used (Takeda Vitamin & Food USA, Orangeburg, NY). Xanthan (TICAXAN®) was kindly supplied by TIC Gums (Belcamp, MD); WPI (Hilmar 9400) by Hilmar ingredients (Hilmar, CA). The WPI used in this study consisted of 93.4% protein (% dry basis), Lactose (0.2%), fat (0.6%), moisture (4%), and ash (2.6%). Green bell peppers and mushrooms were purchased at a local market 24 hours before treatments.

6.2.2 Coating Solution Formation

The formulations are based on 5% (w/v) WPI and 0.5% total polysaccharide (xanthan and curdlan). The components were solubilized in deionized water, with 2 h stirring at room temperature, followed by an overnight storage at 4°C to allow complete hydration of biopolymers. Solutions were centrifuged at 3000 rpm for 15 min to eliminate air bubbles and impure materials, and then heated for 30 min in a 90°C shaking water bath to denature the WPI. Glycerol was added as a plasticizer to the cooled heat-denatured solution at a ratio of polymer: glycerol (70: 30).

6.2.3 Coating Procedure

Mushroom and bell pepper pieces were dipped into the coating solution for 3 min, then the excess coating materials were removed by draining for 20 min under the laminar airflow hood. Coating and draining procedures were repeated twice. Coated samples were placed in the zip-lock plastic bags and stored at -20°C for 10 days.

Thawing of the samples was done for 1 h at room temperature (26±2°C) before analysis. Samples dipped in distilled water following the same procedures were used as controls.

6.2.4 Color Measurements

Color of the samples was determined with a HunterLab ColorFlex Calorimeter (Hunter Associated Lab, Inc., Reston, USA) and L^*, a^*, and b^* values were measured. L^* describes the lightness of the sample, a^* intensity in red ($a^* > 0$), and b* intensity in yellow ($b^* > 0$). Values were reported as ΔL^*, Δa^*, and Δb^*, which are the differences of values of the fresh samples before freezing and after freezing and thawing. The instrument was standardized each time with a white and a black ceramic plate. Samples were scanned at three different locations to determine the average L^*, a^*, and b^*. The whiteness of the mushroom samples was also reported. Whiteness was calculated according to the following formula:

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

6.2.5 Weight Loss

Frozen samples were laid over an absorbent paper and thawed for 1 hour at room temperature. Weight loss (WL) was calculated by the following equation, where w_1 is the weight of the fresh coated sample before freezing and w_2 is the weight of the coated sample after thawing:

$$WL = (w_1 - w_2)/w_1 * 100$$

6.2.6. Texture Analysis

Texture analysis of mushroom and bell pepper was determined by a puncture penetration test, using a TA.XT2i Texture Analyzer (Texture Technologies, Inc., Scarsdale, NY). For the penetration test in the mushroom, a 2 mm diameter punch probe with a 5-Kg load cell was applied for measuring the necessary force to puncture the mushroom cap from the top to a 15-mm depth (test speed: 1.5mm/s). Firmness was defined as the maximum force used in the force vs. time curves. For the puncture test in the bell pepper, a knife blade probe was used to measure the maximum amount of force (N) needed to puncture the pepper samples (test speed: 1.5 mm/s; distance: 6 mm).

6.2.7 Statistical Analysis

Experiments were carried out in triplicate for each treatment and results were reported in Mean±SD. The statistical significance of observed differences between treatment means was evaluated by analysis of variance (ANOVA), and the Duncan test was used to compare treatment means ($P< 0.05$) using IBM SPSS, Statistics 21.

6.3 Results and discussion

6.3.1. Weight Loss

The weight loss of frozen-thawed mushroom and bell pepper are shown in Table 6.1. As seen in this table coating significantly ($P< 0.05$) reduced the weight loss of frozen-thawed samples. Best results were obtained when samples were coated with a mixed coating solution of WPI, xanthan, and curdlan.

Table 6.1. Weight loss of frozen-thawed mushrooms and bell peppers after freezing (-20°C) for 10 days.

Sample	Mushroom	Bell pepper
Control	45.74± 3.14[a]	9.59±1.13[a]
Coated with WPI	13.63± 0.93[b]	8.27±0.67[a]
Coated with WPI-XC	9.32±1.22[c]	5.39±0.35[b]

Different letters within a column indicate significant differences at $P< 0.05$

Coating mushrooms with WPI and WPI-XC showed excellent ability to reduce the amount of weight loss to 70% and 80%, respectively. Regarding green bell peppers, the WPI-XC coating solution significantly reduced the weight loss, but WPI was not effective in weight loss reduction. A previous study, which compared the effect of different coating solutions on the drip loss of frozen vegetables, reported that guar and xanthan gum were effective in reducing the drip loss in frozen vegetables, but dairy proteins (sodium caseinate and whey protein concentrate) were not effective (Downey, 2002).

Previous studies on coating of fresh mushroom reported similar result, which relative weight loss of coated mushrooms (with alginate-based solution) during 19 days of storage was lower than uncoated samples. (Hershko & Nussinovitch, 1998).

6.3.2. Color measurements

The effect of coating with WPI and WPI-XC on the color of mushroom during storage at -20°C for 10 days is shown in Table 6.2. Mushrooms are one of the most

easily browning vegetables due to enzymatic and chemical oxidations, and enzymatic browning reaction is the most detrimental factor in the quality loss of frozen mushrooms (Coggins & Chamul, 2006). The changes of L^*, a^*, and b^*, before freezing and after thawing frozen samples (ΔL^*, Δa^*, and Δb^*) is reported. The ΔL^* of all treatments was negative, showing the lightness of the mushroom diminished during the storage period. The magnitude of ΔL^* in the control sample is higher compared to coated mushrooms. Therefore, coating was capable of delaying the changes of the lightness and maintaining a better appearance in the mushroom. The amount of a^* of all mushroom samples increased during storage and Δa^* in the control sample was higher than in the coated samples. The greater a^* value can be related to the increasing enzymatic browning during the storage period (Eissa, 2007). Since mushrooms are whitish, changes in the whiteness (W) and L^* are an important factor in our perception of their freshness and quality. Previous studies have also reported that the changes of color in coated mushrooms during the storage period were less compared to uncoated samples (Hershko & Nussinovitch, 1998; Eissa, 2007).

Table 6.2. HumterLab colorimetric parameters of mushrooms**.**

Sample	Δ*L**	Δ*a**	Δ*b**	ΔW
Control	-37.52± 2.28 [a]	8.53±0.72 [a]	5.15±0.41 [b]	-36.1±1.71[a]
Coated with WPI	-30.84± 1.54 [b]	7.3±0.33 [b]	5.01±0.34 [b]	-30.02±1.93[b]
Coated with WPI-XC	-24.23± 1.82 [c]	6.68±0.36 [c]	9.75±0.49 [a]	-25.99±2.04[c]

Δ*L*, Δa*, and* Δ*b**are the changes of parameters before freezing and after thawing frozen samples
Different letters within a column indicate significant differences at $P< 0.05$
Negative sign in table signifies that the values decreased after freezing and thawing the samples

Table 6.3 displays the changes of *L**, *a**, and *b** before freezing and after thawing in frozen bell peppers. In all samples the magnitude of *L**, *a**, and *b** decreased after freezing and thawing. The most important color related to our perception of bell peppers is green, which is attributed to the presence of chlorophyll. The scale *a** is the hue for red color vs. green, where a positive number indicates red and a negative number indicates green. Bathochromic shifts from a green to a red hue observed after freezing and thawing can be associated with the degradation of chlorophyll. The largest decrease in greenness was observed in the control sample (higher Δ*a**), showing that the loss of the chlorophyll was higher in this sample. Thus, coating can delay the changes of color in frozen bell peppers, which might be due to the inactivation of the chlorophyllase, which is responsible for the catabolism of chlorophyll. Ochoa-Reyes et al. (2013) reported similar results: coating bell peppers with gum Arabic could reduce the changes of color during the storage of the products at room temperature for ten days.

Table 6.3. HumterLab colorimetric parameters of bell peppers.

Sample	ΔL^*	Δa^*	Δb^*
Control	-2.87±0.19[a]	-1.81±0.13[a]	-7.53±0.82[a]
Coated with WPI	-2.05±0.14[c]	-1.35±0.1[b]	-2.64±0.21[c]
Coated with WPI-XC	-2.36±0.11[b]	-0.98±0.08[c]	-5.26±0.27[b]

ΔL^*, Δa^*, *and* Δb^* are the changes of parameters before freezing and after thawing frozen samples
Different letters within a column indicate significant differences at $P< 0.05$
Negative sign in the table signifies that the values decreased after freezing and thawing the samples

6.3.3. Texture Analysis

Figure 6.1 compares the maximum force in the puncture penetration test in mushroom (a) and bell pepper (b). As seen in Figure 6.1.a the firmness of mushrooms increased significantly after freezing and thawing. It reduced from 10.21 N in fresh mushroom, to around 3 N in coated and uncoated frozen-thawed samples (≈70% decrease). In Figure 6.1.b the maximum force needed to puncture the fresh pepper was 133 N, while in frozen-thawed samples including uncoated, WPI-coated, and WPI-XC coated samples were 34.33, 42.3, and 69.48 N, respectively. Therefore, the results showed that coating mushroom with WPI and WPI-XC solution used in this study was not effective in delaying the loss of firmness in frozen mushroom. Whereas, in bell pepper coating samples with WPI-XC solution could significantly increase the firmness of the frozen-thawed product compared to uncoated and coated samples with WPI alone. There was no significant ($P< 0.05$) difference in the firmness of the control pepper and sample coated with WPI.

Cellulase and pectinase are the main enzymes responsible for the textural changes in vegetables. Freezing results in dissolution, depolymerization, and destruction of cell wall composition.

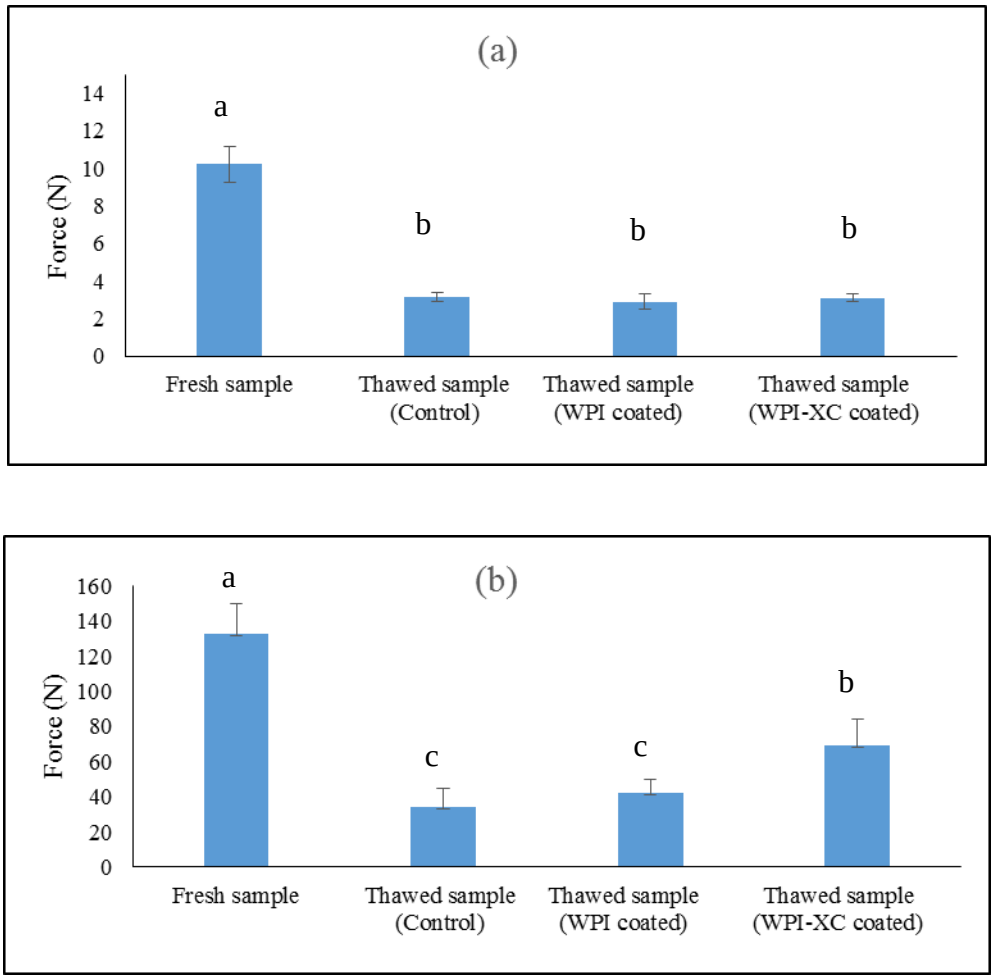


Figure 6.1. Comparison of maximum force needed to penetrate into fresh and frozen-thawed samples for mushroom (a), and bell pepper (b)

6.4. Conclusion

Freezing is a common process for the long-term preservation of vegetables. However, during frozen storage, physical, chemical, and nutritional changes occur; thus the quality of the final product is unlike the fresh product in flavor and color characteristics. Results in this study showed that coating mushroom and bell pepper with a WPI-XC mixed solution was an effective method to reduce the quality changes during freezing process. Mushroom samples coated with WPI-XC showed less weight loss (five times lower than the uncoated sample) with a lower ΔL^*, which is the changes of the lightness during storage in thawed samples: ΔL^* in coated mushroom with WPI-XC was 35.42% lower compared to the uncoated sample. Coating the green bell peppers with WPI-XC was capable of reducing the weight loss and retaining the greenness better than the other two samples. The weight loss in the sample coated with WPI-XC was 1.7 times lower than that of the uncoated sample with a less changes of a^* (0.98% in coated with WPI-XC sample compared to 1.81% in the uncoated sample). Regarding the effect of coating on the texture of frozen thawed samples according to the puncture penetration tests, no improvement was observed in the coated mushrooms compared to the uncoated ones, while in the bell pepper, coating with WPI-XC was able to improve the firmness of the pepper.

The textural quality of vegetables declines upon freezing and thawing, resulting in the softening of the product; such loss of textural integrity represents the main defect of vegetable products. During freezing storage, the color of the frozen mushrooms gradually turns greenish brown or dark brown, an unacceptable appearance to consumers. Furthermore a dark liquid exudes from the frozen-thawed

mushroom, which can darken entire dishes in meals containing mushrooms. Using the mixed WPI-XC solution to coat mushrooms and bell peppers showed excellent capacity to reduce the expulsion of water from the frozen-thawed samples, and consequently, in reducing the weight loss. Therefore, coating mushroom and bell peeper with the WPI-XC solution can benefit manufacturers by enabling them to produce a product that looks better and has greater appeal to consumers. Further studies are required to investigate the efficacy of the WPI-XC coating solution in improving the texture of frozen-thawed vegetables by altering the ratios and/or concentrations of these biopolymers, and other conditions, such as pH.

Chapter 7: Conclusions and Recommendations

Addition of xanthan-curdlan hydrogel to WPI solution, showed excellent ability to improve freeze-thaw stability and water holding capacity of the heat-induced whey protein gels. Total concentration of 0.5% gum in the gel formulation, was able to eliminate syneresis up to four freeze-thaw cycles. It also increased the storage modulus (G') of the gels before freezing and after freeze-thaw cycles, and minimized the discrepancies of G' in the frequency sweep tests, indicating the improvement of the stability in the system over 5 FTCs. Through scanning electron microscopy it was confirmed that XCHC could behave as a pore-forming agent; it increased the porosity, decreased the pore size, and improved the gel's water retention over multiple freeze-thaw cycles. FTIR results showed that addition of XCHC to WPI solution increased the intensity of peaks associated with the β-sheet, indicating heat-induced WPI-XCHC mixed gels had a higher degree of intermolecular hydrogen bonding compared to the pure WPI gel.

Dynamic rheological measurements over the pH range of 4-7, revealed that storage modulus and gelation temperature of the heat-induced mixed gels were strongly pH-dependent. XCHC increased the storage modulus of the gels. Over the pH range of 5-7, gels showed phase-separated network, while at pH 4 gels with the peculiar networks were formed. Manipulation of the interactions between WPI and XCHC by controlling pH enables food scientists to create unique and fabricated formulation with superior sensory and physiochemical properties, more acceptable to consumers.

Results obtained from the analytical experiments (zeta potential, particle size, and turbidity) provide us some useful knowledge about the interactions of whey protein and XCHC in aqueous solutions: soluble or insoluble complexes depending on the pH can be formed. Insoluble complexes were formed at pH values below the isoelectric point of the whey protein where the protein and polysaccharide had opposite electrical charges; soluble complexes were formed at pH≈5, where XCHC was negatively charged and whey protein had a little net charge (due to the positively charged patches on the surface of the whey protein); no complexation was observed at pH values above 6, due to the strong electrostatic repulsion between the biopolymers. These findings could be useful in designing novel food ingredients with unique functionalities.

Finally the efficacy of a mixed solution containing whey protein isolate, xanthan, and curdlan as an edible coating in mushroom and green bell pepper were investigated. The results showed that WPI-XC was capable of decreasing some quality changes during the freezing storage; it was particularly effective in reducing the moisture loss in the frozen-thawed vegetables.

The ability of XCHC to strengthen the rheological properties of heat-induced WPI gel and to eliminate syneresis suggests that it could explore the possibility of new applications in novel food formulation, in particular in the frozen food products. To narrow an optimum range of usage for the mixed WPI-XCHC in the food industry, the following recommendations are offered for the related research area:

- Further studies on the effect of XCHC on other major food components such as soy protein isolate and casein to improve freeze-thaw stability.

- Further studies on the effect of sugar, as one of the main components in the frozen desserts, on the freeze-thaw stability of WPI-XCHC heat-induced gels.
- Further studies on the efficacy of insoluble complexes of WPI-XCHC, as a meat substitute in the frozen products, and to investigate the freeze-thaw stability of these complexes over multiple freeze-thaw cycles compared to other protein polysaccharide complexes used for this purpose.
- Further studies on some modification methods, such as microfluidization, in the XCHC to produce WPI-XC complexes with an appropriate structure applicable as a protein-polysaccharide-based fat replacer in the frozen products.
- Further studies to investigate the effect of WPI-XC as an edible coating on the quality changes of meat and seafood products over multiple freeze-thaw cycles.

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