FUNCTIONALIZATION OF WHEY PROTEIN BY REACTIVE SUPERCRITICAL FLUID EXTRUSION (RSCFX)

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FUNCTIONALIZATION OF WHEY PROTEIN BY REACTIVE SUPERCRITICAL FLUID EXTRUSION (RSCFX)

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Reactive supercritical fluid extrusion (RSCFX) is a novel integrated process for controlled chemical reactions and continuous generation of expanded extrudates of modified functionalities. Twin screw extruders are ideally suited for highly viscous materials due to their excellent mixing abilities which help maximize reaction rates. Beyond their superior nutritional qualities, whey proteins are also utilized for thickening, stabilization and emulsification of food formulations following pH adjustment and heat treatment to induce protein denaturation and aggregation. Creating cold-gelling and thickening functionalities in whey protein for use in food system where heating is undesirable remains a challenge. Also, replacing starch-based thickeners with a whey protein ingredient may be attractive to diet-conscious consumers. The aim of this work was to alter and quantify the functional properties of whey proteins by RSCFX processing to create novel dairy ingredients for food applications. Texturized whey protein concentrate (TWPC) at acidic condition (pH 3.0) were made by RSCFX and the effects of addition of starch, calcium (0.3 and 0.6 %, w/w), and extrusion temperature (50, 70 and 90 °C) on selected physicochemical properties of the modified protein were evaluated. TWPC exhibited 200- to 300-fold higher viscosities than non-texturized WPC at various concentrations (6–26%, w/w TWPC) and formed cold-set gels at 20 % (w/w) upon reconstitution in water. Starch-containing samples (TWPC-S) were less soluble, consisted of larger protein aggregates (1.527 μm), but had 1.2–1.4-fold higher apparent viscosity than TWPC alone because of synergistic interactions between the two biopolymers. TWPC without the starch and at lower calcium level (0.3%) had

smaller protein aggregates (0.996 μm) with higher solubility. TWPC alone extruded at 50 °C (TWPC-50) and 70 °C (TWPC-70) formed soft-textured aggregates with high solubility in water (77–79 %) than that extruded at 90 °C (TWPC-90) with a solubility of 24%. Total free sulfhydryl contents and solubility studies in selected buffers indicated that non-covalent interactions were prevalent in stabilizing the TWPC aggregates. TWPC extruded at 90 °C showed an increase in aromatic hydrophobicity and a decrease in aliphatic hydrophobicity indicating changes in protein structures. Secondary gelation occurred in TWPC-50 and TWPC-70 when the cold-set gels were heated to 95 °C, while TWPC-90 showed excellent thermal stability. Factors such as the degree of protein denaturation, exposure of hydrophobic groups, and cross-linking influenced the intermolecular associations and improved the cold-set and second-stage heat-induced gelation of TWPCs. Compared to non-texturized WPC, TWPC formed stable oil-in-water emulsions at lower protein concentrations. However, the presence of starch in TWPC impeded emulsifying properties. TWPCs were able to form cold-set emulsion gels containing 40 to 80% (w/w) that were stabilized by 4 to 12% (w/w) protein and had smaller mean droplet sizes as opposed to the non-texturized WPC. TWPC-90 emulsions showed excellent stability during storage (30 days at 4 and 25 °C) and heating (70–90 °C for 20 min) due to the extensively denatured proteins. Heat treatment ≥ 80 °C of non-texturized WPC and TWPC-70 emulsions increased the droplet sizes and loss moduli (G'), indicating emulsion destabilization due to aggregation of native proteins. TWPC emulsions had higher adsorbed proteins (6.0–23.3 mg/mL) in contrast to the non-texturized WPC emulsions (2.17–6.33 mg/mL). SDS-PAGE of the adsorbed TWPC showed greater intensity of α-la (α-lactalbumin) and the presence of high molecular-weight protein aggregates. The adsorbed proteins of TWPC-70 emulsions underwent time-dependent polymerization, but they remained stable in TWPC-90-based emulsions.

The superiority of TWPC stabilized emulsions may be due to combinations of a stable protein gel matrix that formed the continuous phase of emulsion, greater surface hydrophobicity,

and intra-film protein polymerization that conferred strength to the protein interfacial layer. TWPC-90 that contains a higher degree of denatured protein offers the best potential to serve as a novel, whey protein-based food emulsifier and stabilizer. The RSCFX process provides a new approach by which functional characteristics of TWPC ingredient may be advantageously designed by altering the formulation composition and extrusion operating parameters. These new ingredients may be utilized in different products requiring targeted physicochemical functionalities and a cleaner, all-dairy label.

BIOGRAPHICAL SKETCH

The author was born in Kota Bharu, Kelantan, Malaysia to Mustapha Jusoh and Salehah Mat Sin. She received her Bachelor of Technology in Food Technology from Universiti Sains Malaysia in 2003. Right after graduation, she joined the Faculty of Food Science and Technology of University Putra Malaysia as academic staff. She received Government Scholarship from Ministry of Higher Education, Malaysia to pursue her Master of Science in Food Science at University Putra Malaysia, and graduated in April 2008. She received another scholarship form Ministry of Higher Education to pursue her PhD degree. In August 2008, she enrolled in the Field of Food Science, Cornell University under the guidance and direction of Prof Syed Rizvi.

To my mom, dad, and siblings, for your love and support

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CHAPTER 1

GENERAL INTRODUCTION

Whey protein (WP) is a multi-component protein mixture of high nutritive values. The unique functional properties of WP make it a highly useful food ingredient in a wide array of applications including thickening, gelling, emulsifying and foaming of food matrices (Dalgleish, 1996; Morr & Ha, 1993). The functional properties of WP vary depending on their intrinsic factors such as size and shape of protein aggregates, surface hydrophobicity, and molecular flexibility, which are themselves affected by their environmental conditions such as pH, ionic strength, and temperature. The degree of exposure of reactive amino acids such as hydrophobic and thiol groups during heating determines the functionalities of the protein. Extensive protein aggregation impairs the solubility and interfacial properties of WP (deWit & Klarenbeek, 1984; Moro, Gatti, & Delorenzi, 2001). Increasing content of salts increases gel strength, which then decreases upon further addition of salts (>30 mM $CaCl_2$), due to the formation of larger WP aggregates (Mudgal, Daubert & Foegeding, 2011; Schokker, Singh, Pinder & Creamer, 2000). The presence of calcium ions may induce fat droplet aggregation due to the reduction in electrostatic repulsion between fat droplets (Kulmyrzaev, Chanamai & McClements, 2000). Moreover, other component such as starch enhances the gel strength of mixed WP-starch systems due to synergistic role of both polymers (Shim & Mulvaney, 2001), but it impairs emulsion stability due to the non-adsorbing nature of the

biopolymer, leading to phase separation (Cao, Dickinson & Wedlock, 1990). Therefore, improving specific functionality requires a careful modulation of other physicochemical properties of the protein.

The high temperature treatment (>65 °C) applied to achieve required structural modifications and functionalities of WP is not a suitable option for certain product applications. Therefore, a process to obtain WP ingredient that thickens or gels at room temperature (20–40 °C)—cold-set gels—is highly desirable. The process combines a controlled thermal denaturation (70–90 °C for 5–120 min) of WP solution under neutral pH and low ionic strength to form soluble aggregates, followed by cold-set gel formation through the addition of salt or reduction of pH (Alting, Hamer, de Kruif, & Visschers, 2000; Barbut & Foegeding, 1993; Cavallieri & da Cunha, 2008). The cold-set thickening and gelling ingredients exhibited improved water-holding capacity and higher strength than those produced by conventional heat-induced methods and are stable over a wide range of temperature and pH values. The modified proteins provide food industry with more alternatives in product formulations including superior nutritional values when compared with polysaccharide-based stabilizing agents, such as pregelatinized starch and carrageenan (Hudson, Daubert, & Foegeding, 2000; Alting *et al*., 2000; Resch, Daubert, & Foegeding, 2004). The protein ingredients also have a high potential for product applications containing heat-labile compounds (Britten & Giroux, 2001) or to increase bioavailability of bioactive components such as iron (Remondetto, Paquin, & Subirade, 2002).

Although the WP-based cold-set thickening agent appears to have a great promise in food applications, there are only a few techniques that have been proposed to date to

produce the cold-set WP thickening ingredient in powder form. However, most of the processing techniques proposed require high temperature, long or multiple processing steps, in which, some of the parameters applied are known to have detrimental effects on emulsifying and foaming properties of proteins (Dissanayakee, Kelley, & Vasiljevic, 2010; Hudson *et al*., 2000). A higher concentration of the protein ingredients is also required to achieve thickening or gelling capacity similar to traditional hydrocolloids, such as carrageenan, xanthan gum or starch (Hudson & Daubert, 2002; Resch *et al*., 2004).

In addition to formation and stabilization of emulsions, WP is also used to modify and improve the organoleptic properties and overall acceptability of food, via utilization of emulsion gels. Heat treatment has been widely used to convert WP emulsions into heat-set gels (Dickinson & Chen, 1999; Kinsella, 1984). The stability of emulsions during heat treatment, storage and final consumer use is a highly important characteristic for their utilization in foods. However, heating at a specific temperature/time combination affects the stability of emulsions due to the aggregation of fat droplets. The aggregation of the droplets depends on the thickness of protein layer, amount of adsorbed and non-adsorbed proteins, hydrophobicity of both protein molecules and emulsion droplets, and aqueous phase conditions such as pH and ionic strength (Demetriades, Coupland, & McClements, 1997; Dickinson & Parkinson, 2004; Euston, Finnigan & Hirst, 2000; Sliwinski, Roubos, Zoet, van Boekel & Wouters, 2003). Furthermore, the high temperatures needed for gel formation prevent incorporation of thermo-labile compounds into the emulsion gel systems. Therefore, cold-gelation of WP-stabilized emulsions has been reported as one of the possible alternatives to overcome these

limitations (Britten & Giroux, 2001; Sok Line, Remondetto, & Subirade, 2005; Ye & Taylor, 2009).

Understanding the factors influencing the molecular properties of WP is critical for developing and utilizing modified protein ingredients. Therefore, this research was designed to understand and achieve a better control of the physicochemical properties of WPC through the use of a reactive supercritical fluid extrusion process (RSCFX). The process was used as a controlled bioreactor by utilizing a precise control of temperature, shear, pressure and internal environments created by introduction of SC-CO_2 that results in controlled denaturation and aggregation of WP. The overall objective was to understand the effects of extrinsic factors such as added starch and calcium and extrusion temperatures on the functionalization of texturized WPC. This study also aimed to create a new cold-setting WP gels with improved emulsifying properties that could be used as ingredients in food applications.

Based on the outlined objective, the chapters in the dissertation are organized in the following manner:

Chapter 1 gives a general introduction of the thesis.

Chapter 2 provides a brief overview of the functionalities of WP in general and of cold gelation and texturization of proteins in particular.

Chapter 3 describes the effects of varying feed formulations by addition of starch and variation in calcium levels during the RSCFX process on the physicochemical properties of TWPC. The ability of the texturized powders to impart cold-set thickening and gelling functionalities was investigated using rheological techniques.

Chapter 4 details the study on the ability of TWPC to form cold-set emulsion gels at different oil levels. The stability of the gels was studied as a function of storage time and temperature, and heat treatment using microscopic observations. The surface hydrophobicity of modified protein was also investigated as a factor to enhance the emulsifying properties.

Chapter 5 describes the effects of extrusion temperatures on the degree of denaturation of WPC and its influence on the resulting functionalities. The steady shear rheological method was used to compare the cold-gelation capacity of TWPC obtained at low and high temperatures. The changes in rheological characteristics of the protein at high temperature and molecular interactions involved in stabilizing the aggregates were also investigated.

Chapter 6 reports the storage and heat stability of cold-set emulsions made from TWPCs produced at different extrusion temperatures. The effects of the amount of adsorbed proteins and their changes during storage and thermal treatment on the stability of emulsions were investigated using electrophoretic method.

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CHAPTER 2

LITERATURE REVIEW

2.1. Compositions of whey protein

Bovine milk is comprised of two major protein types, whey and casein. Whey protein (WP) is defined as the protein fraction that remains soluble in milk serum after removal of casein during cheese manufacture. Acid whey, with a pH less than 5.1 is the result of casein coagulation via acidification, while sweet whey, with a pH of at least 5.6 is the remaining liquid of rennet-coagulated milk (Fox & McSweeney, 1998). The proteins of whey represent about 20% of total milk proteins. The liquid whey contains approximately 0.6% protein and 93% water. The utilization and/or disposal of whey have been major concerns as it contains valuable constituents that should not be wasted.

Whey protein (WP) is a compact globular protein consisting of 55% β-lactoglobulin (β-lg), 24% α-lactalbumin (α-la), ~5% bovine serum albumin (BSA) and 12% immunoglobulin (Ig). Minor proteins include lactoferrin, lactoperoxidase, enzymes, protein components of the milk fat globule membrane (MFGM), proteose-peptone components and glycomacropeptide (only found in sweet whey) (deWit & Klarenbeek, 1984). Both β-lg and α-la have major impact on functionality of WP when used as an ingredient. The properties of WP are shown in Table 2.1.

Table 2.1. Distribution and characteristics of whey protein fractions.

Components	Approx. concentration		Approx. molecular weight (Da)	Groups per mole.			
	% of skim milk protein	g/L		pI	P	-S-S-	-SH
β-lg	7.0-12.0	3.6	18 300	5.2	0	2	1
α-la	2.0-5.0	1.7	14 200	4.2-4.4	0	4	0
Ig	1.5-2.5	0.6	160 000 – 900 000	5.5-8.3	0	Variable	
BSA	0.7-1.3	0.4	66 000	4.7	0	17	1
Proteose-peptone	2.0-4.0	0.7	4 000 – 40 000	3.7	0.5-2.0	0	1

(adapted from Brunner, 1976; deWit & Klarenbeek, 1984)

β-lg consists of 162 amino acid residues having a molecular weight (MW) of 18.4 kDa (Morr & Ha, 1993). The protein is classified in lipocalin superfamily of proteins that is folded as eight strands of antiparallel β-sheet that forms a central cavity, the calyx, which is capable of binding small hydrophobic molecules such as retinol, short-chain fatty acids, alkanes, aliphatic ketones, and aromatic compounds. These properties suggest that β-lg may involve in transportation of lipid-soluble biological components (Perez & Calvo, 1995; Wu, Perez, Puyol, & Sawyer, 1999). The monomer of β-lg possesses five cysteine (Cys) residues that participate in the formation of two disulfide bonds (S-S) at Cys66 to Cys160 and Cys106 to Cys119; and a thiol (-SH), at residue

Cys121 that is normally buried internally in the native molecule. The secondary structure of the native protein consists of 10-15% α-helix, 43% β-sheet and 47% unordered structure, including β-turn. The tertiary structure of β-lg shows a very compact globular structure, in which β-sheet occurs in β-barrel structure, stabilized by two disulfide bonds (Boye, Alli, & Ismail, 1996). The native protein molecule adapts different quaternary structures depending on protein concentration, pH and nature of ions (Verheul, Pedersen, Roefs, & de Kruif, 1999). In the native form and in pH range between 5.2 and 7.5 β-lg exists as a dimer held together by non-covalent interactions, whereas at $3.5 < pH > 7.5$, the dimer dissociates into monomers. Octameric form of β-lg exists in the pH range of 3.5 and 5.2 (Verheul, Roefs, & de Kruif, 1998).

β-lg is the most heat labile WP, undergoes a time/temperature-dependent denaturation above 65 °C, accompanied by extensive conformational transition and loss of α-helix and β-sheets structures due to dissociation of hydrogen bonds that stabilize the native structure. This process facilitates the formation of new intermolecular β-sheets arrangements via thiol/disulfide interchange reactions, and also enhances the exposure of hydrophobic residues (Kinsella, 1984; Roefs & de Kruif, 1994). A reversible conformational change occurs below 70 °C but it undergoes irreversible denaturation and polymerization at higher temperatures. The protein is resistant to thermal changes at low pH values (deWit & Klarenbeek, 1984).

α-lactalbumin (α-la) is globular, calcium metallo-protein having 123 amino acid residues with a MW of 14 kDa. The protein participates in the regulation of lactose biosynthesis by forming a part of the enzyme lactose synthetase and binds with galactosyl transferase to modify its substrate (glucose) to lactose during lactation. α-la shares 40%

sequence homology with lysozyme. It consists of four α-helices and two β-strands with a loop-like chain (Farrell *et al*., 2004). It has the lowest denaturation temperature (~61°C) of the WPs, but is considered the most thermostable due high capability of renaturation (>90% thermoreversible) (Morr & Ha, 1993; de Wit & Klarenbeek 1984). The Cys residues that form four disulfide bonds are mainly responsible for the reversible conformational change upon heating. α-la does not form aggregates by itself when heated above 70 °C due to the lack of free thiol (-SH) group. Certain carboxyl groups such as Asp residues of the molecule are involved in the binding of calcium ions which contribute to its higher heat stability. The removal of bound calcium ions by heating at high temperatures (>85 °C) (Morr & Ha, 1993) or acidification to pH 4.0 renders α-la more sensitive to heat and reduces its ability to renature (Kronman, Andreotti, & Vitols, 1964; Relkin, 1996). Chelation of the protein ions by 0.1 M EDTA at 20 °C decreases its denaturation temperature (Bernal & Jelen, 1984).

2.2. Commercial production of whey protein products

Whey can be processed into food ingredients by simple drying, or the protein content can be further increased by removing other components such as lipids, minerals and lactose. With the advent of industrial ultrafiltration and chromatographic methods, recovery and fractionation of WP in their native forms has become possible. The production technology of WP is classified into two major categories: membrane technology that includes cross-flow microfiltration (MF) and ultrafiltration (UF), and ion-exchange chromatography (IE) (Fox, 2003). The most important commercial WP

products are whey protein concentrates (WPC) and whey protein isolates (WPI). Others whey products include whey protein fractions (β-lg and α-la rich fractions), protein hydrolysates, lactoferrin, lactoperoxidase and casein glycomacropeptide (Abd El-Salam, El-Shibny, & Salem, 2009; Morr & Foegeding, 1990).

WPC contains between 34 to 80% proteins in addition to other milk components such as lactose and fat and is produced via ultrafiltration/diafiltration of whey followed by spray drying. Ultrafiltration of whey removes the lactose and minerals, while diafiltration removes more of the components, resulting in WPC of different composition and functionalities. WPI generally contains 90 to 96% proteins and is often devoid of lactose and other small molecular weight (MW) components. The protein is prepared by either ion exchange or microfiltration processes followed by spray drying. Depending on the process used, WP products tend to contain various levels of lipids, lactose and minerals as well proteins with different degrees of denaturation and aggregation. These variations have a major influence on the emulsifying and other functional properties of the WPs (Morr & Foegeding, 1990). All the soluble WPs are concentrated in the microfiltration or ultrafiltration processes, whereas in the ion-exchange chromatography some proteins, e.g., glycomacropeptide (GMP), lactoferrin and small peptide fragments are lost. These differences in composition lead to variations in nutritional and functional properties of WPC and WPI.

2.3. Physicochemical and functional properties of whey proteins

WP is widely used in food products because of the high nutritional values and technological properties. In general, the functional properties of WP can be classified into two main groups: hydration-related and surface-related properties. Hydration related properties include dispersibility, solubility, swelling, viscosity, and gelation. Surface related properties include emulsification and foaming (Bryant & McClements, 1998; Morr & Ha, 1993).

Protein molecules must undergo a series of physicochemical reactions that include dispersion, dissociation, molecular unfolding and rearrangement, diffusion, adsorption, and interaction in a specific sequence of events in order to provide functionality. The functional properties of WPs are governed through multiple chemical and physicochemical properties that result from complex interactions between intrinsic (amino acid composition and sequence, conformation, molecular size, flexibility, net charge and reactivity of sulfhydryl and hydrophobic amino acid residues) and extrinsic (pH, ionic strength, temperature, concentration of emulsifiers) factors (deWit, 1998; Kinsella &Whitehead, 1989). Some of the physicochemical properties of WP are inter-related. For example, heating of WP results in gelation of the protein attributed to the formation of networked protein aggregates. However, extensive aggregation reduces protein solubility in water and impairs its interfacial properties (deWit & Klarenbeek, 1984; Dissanayakee, Kelley, & Vasiljevic, 2010). Therefore, several modification methods have been explored to develop new and enhanced functionalities in order to expand the utilization of WP.

2.4. Modification of whey proteins

Multiple attempts have been made to improve the functionality of WP via modification processes that can be accomplished by chemical, enzymatic and physical techniques (Howell, 1996; Hudson, Daubert, & Foegeding, 2000). Chemical modification includes alteration of amino groups (acylation, alkylation) and carboxyl groups (esterification, amidation), as well as covalent attachment of amino groups, phosphates and carbohydrate (Howell, 1996). Conjugation of protein via covalent bonding with polysaccharides and smaller sugars substantially improves the functional properties of WP including interfacial properties, solubility and heat stability.

Enzymatic modification of food proteins involves controlled proteolysis to yield a mixture of peptides. The functional properties of protein are altered depending on specificity of enzymes, substrate and enzyme concentrations, pH, ionic strength and temperature. Transglutaminase has been used to enhance physicochemical properties of milk proteins by introducing new intra- and inter-molecular crosslinks, decreasing molecular weight and exposure of hydrophobic residues. The enzymatic treatment leads to favorable changes in solubility and gelation properties of WP (Dickinson & Yamamoto, 1996; Eissa, Bisram, & Khan, 2004).

Physical modification of protein may involve thermal treatment, high pressure and extrusion processing. Thermal treatment that results in partial denaturation of protein improves functional behavior of WP, while complete denaturation usually impairs the solubility and other functional properties (deWit & Klarenbeek, 1984). High hydrostatic pressure induces modification of protein structures by changing the balance of

intramolecular and solvent-protein interactions such as disruption and reformation of hydrogen bonds and rupture of hydrophobic bonds (Hayakawa, Linko, & Linko, 1996; Lopez-Fandino, 2006). Pressurization of WP alters the interfacial and gelation properties of WP (Galazka, Ledward, Dickinson, & Langley, 1995). Extrusion processing texturizes globular proteins by shearing and stretching them into aligned or entangled fibrous networks with new functionalities. The process imparts changes in the structural conformation of proteins resulting in enhanced gelation behavior (Queguiner, Dumay, Salou-Cavalier, & Cheftel, 1992; Walsh & Carpenter, 2003).

2.5. Heat-induced denaturation and aggregation of whey proteins

The mechanisms and kinetics of heat-induced WP denaturation and aggregation have been extensively studied. Thermal behavior of WP is dominated by β-lg, the major protein components of WP (Livney, Corredig, & Dalgleish, 2003; Hoffmann & van Mil, 1999; Roefs & de Kruif, 1994). Denaturation of WP can be described by a two-step process: protein unfolding followed by aggregation. The process causes a major change in the specific native structure of protein, which alters the balance between different forces stabilizing the protein structure including hydrogen and disulfide bonds, electrostatic, dipole-dipole, and hydrophobic interactions (Mulvihill, Rector, & Kinsella, 1990; Morr & Ha, 1993). In the first step, upon heating above ~60 °C, protein molecule partially unfolds, exposing the initially buried hydrophobic and thiol groups. β-lg monomer-dimer equilibrium shifts toward monomers. The irreversible protein aggregation occurs in the second step, predominantly as a result of intermolecular

thiol/disulfide interchange reactions and, to a lesser extent, from non-covalent interactions. The contribution of non-covalent interactions becomes increasingly important when pH values are closer to the isoelectric point (pI), at temperature above >90 °C and/or at higher salt concentrations (Iametti, de Gregori, Vecchio, & Bonomi 1996; McKenzie, Ralston, & Shaw, 1972; Verheul *et al*., 1998).

The formation of disulfide-linked β-lg aggregates in the temperature range of 60–70 ºC and at near-neutral pH has been proposed using kinetic aggregation model based on thiol/disulfide exchange reactions analogous to radical-addition polymerization reaction (Roefs & de Kruif, 1994). The process consists of three steps; initiation, propagation, and termination. The sulfhydryl (-SH) groups act as the radicals, whereas β-lg plays the role of both initiating and propagating monomer. The initiation step involves the activation of β-lg monomer and it consists of a number of reversible reactions follows by an irreversible reaction. In the reversible reactions, the β-lg dimer splits into two monomers. In the latter reaction, the free –SH group of native β-lg (B) that was initially buried is exposed and consequently becomes reactive (B^*) (Eq. 1). The propagation step occurs when the reactive -SH group, B^*, reacts with one of the two intramolecular disulfide bonds of a native β-lg monomer (B) via a sulphydryl/disulfide interchange reaction to form a dimer, B_2^*. An intermolecular disulfide bond is formed and a new reactive free -SH group is now available from the originally non-denatured molecule, leaving a reactive dimer to react with a native monomer (Eq. 2). This step is repeated many times, which finally results in linear aggregates. The propagation step proceeds until termination stage occurs. In the termination step, two reactive –SH group react with

each other and create a new disulfide bond but no free SH group, leading to irreversible aggregation (Eq. 3).

$$B_2 \leftrightarrow \ldots \leftrightarrow B \xrightarrow{k1} B^* \quad (1)$$

$$B + B_i^* \xrightarrow{k2} B_{i+1}^* \quad i \geq 1 \quad (2)$$

$$B_i^* + B_j^* \xrightarrow{k3} B_{i+j}^* \quad i \geq 1 \quad (3)$$

Although the aggregation kinetics of WP is largely determined by β-lg, other WPs such as α-la and BSA may also determine the characteristics of the aggregation process. α-la that does not form aggregates when heated alone will polymerize in the presence of β-lg or BSA. The protein molecules interact with each other and form aggregates through covalent and non-covalent interactions like hydrophobic, ionic and/or der Waals interactions (Hines & Foegeding, 1993; Dalgleish, Senaratne, & Francois, 1997).

2.5.1. Factors affecting denaturation and aggregation of whey proteins

Aggregation of protein affects other functional properties such as solubility, gelation and interfacial properties. The rates of denaturation and aggregation depend on the heating temperatures. Mild heating of WP at 40–60 °C causes reversible changes in β-lg and α-la structures, which may reversibly affect the solubility, whereas at higher temperatures, the denaturation is less easily reversible and leads to increasing growth of aggregates (de Wit & Klarenbeek, 1984; Le Bon, Nicolai, & Durand, 1999). Increasing

heating temperature up to 60–70 °C enhances hydrophobic interactions, which are weakened with further temperature increase (Li-Chan, 1983). Heating β-lg at pH 4.6 from 40 to 60 °C results in intramolecular association (folding) without loss of solubility, while hydrophobic residues in BSA are exposed, resulting in increasing tendency for hydrophobic aggregation (Macritchie, 1973). Enhanced thermal stability of β-lg was observed at acidic pH (pH 3.6–4.5) compared to α-la. However, stability of the protein molecule decreases at neutral or alkaline pHs, causing a loss in solubility due to increased disulfide interchange reactions catalyzed by proton dissociated thiol groups. When acidic WP solutions are heated to even 40 °C , α-la readily denatures to form aggregates, but it does not aggregate above pH 7 (de Wit & Klarenbeek, 1984; Hillier, Lyster, & Cheeseman, 1979; Li-Chan, 1983). At high temperatures (> 95 °C), irreversible denaturation takes place very rapidly, independent of pH, due to the severe degree of changes in intermolecular linkages (Petit *et al*., 2012; Spiegel & Huss, 2001). The residual protein structures remain below 100 °C, but the unfolding does occur between 125-150 °C, probably induced by the cleavage of disulfide bonds (de Wit, 2009).

Higher ionic strength promotes heat-induced denaturation and aggregation of WP by neutralizing their charges and formation of calcium bridges (Bryant & McClements, 1998, 2000b; Schmidt, Illingworth, Deng, & Cornell, 1979). pH affects the distribution of electrostatic charges along the protein polypeptide chains, hence alters the balance of attractive and repulsive intermolecular forces that favor denaturation or aggregation of proteins, thereby affecting their precipitation properties (Vojdani, 1996). At pH values closer to the isoelectric point of the main WP (β-lg, α-la, and BSA) aggregate formation occurs at a lower rate than at higher pH (Hillier et al., 1979; Verheul *et al*., 1998). The -

SH residues are mainly responsible for the formation of thermally induced protein interactions, whereas, Ɛ-amino and carboxyl groups influence the charges that promote or inhibit electrostatic interactions and calcium-protein interactions.

Two types of β-lg aggregates are formed depending on the extrinsic factors prevailing in the system. At temperature lower than 85 °C and pH values close to the isoelectric point or at higher ionic strength, the contribution of non-covalent interactions, mainly hydrophobic, to the overall aggregation mechanism becomes important. Aggregates formed under these conditions are spherical, rigid and denser, with low water holding capacity. Disulfide interactions predominate at high temperatures (≥85 to 95 °C), neutral or acidic pH, low ionic strength, or low calcium content. The aggregates formed are smaller, soft, open, and easily deformable, with a good water holding capacity (de Wit, 2009; Havea, Watkinson, & Kuhn-Sherlock, 2004; Ikeda & Li-Chan, 2004; Nicolai, Britten, & Schmitt, 2011; Spiegel, 1999; Spigel & Huss, 2002; Verheul *et al*., 1998).

Although heat-induced WP denaturation and aggregation during processing is detrimental to the WP functionality, yet denaturation of globular proteins in many cases is essential to "activate" the functionality that is desired for the textural properties of food. The functional properties of WP such as foaming and emulsifying properties can also be altered by heating under defined conditions (de Wit & Klarenbeek, 1984).

2.6. Whey protein gelation

The ability of WP to form gel capable of providing textural properties and holding water, lipids, and other components is the basis of the wide use of WP as a functional ingredient in foods. WP gels can substitute the role of fats in enhancing textural properties of foods (de Wit, 1998; Kinsella & Whitehead, 1989). Controlling protein gelation is an important step in the development of new textures and stability in dairy products and can be induced by a number of extrinsic factors such as heat, acids, pressure, enzymes, and salts (Eissa et al, 2004; Havea *et al*., 2009; Resch, Daubert, & Foegeding, 2005).

2.6.1. Heat-induced gelation of whey proteins

WP gels are mostly formed by heating and are referred to as heat-induced or heat-set gels. Gelation of β-lg occurs at temperature higher than 65 °C and at a sufficiently high (10–12%, w/w) protein level in a three-step process involving protein unfolding, aggregation and association of protein particles via protein-protein and protein-water interactions as shown in Figure 2.1. The three-dimensional protein structure is capable of immobilizing large quantities of water and other ingredients through the formation of intermolecular disulfide, hydrophobic and ionic bonds (Le Bon et al., 1999; Xiong 1992).

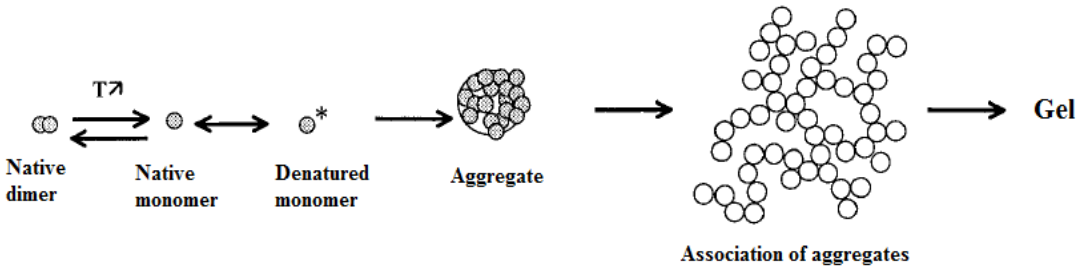

Figure 2.1. Heat-induced aggregation and gelation process of β-lg in aqueous solution at pH 7.0 (Le Bon et al., 1999)

The gelation process depends on the balance of attractive and repulsive forces among the denatured protein molecules during aggregation, which is influenced by the environmental conditions, protein type, protein concentration and processing parameters (heating temperature, time and rate) (Bowland & Foegeding, 1995; Leksrisompong, Lanier & Foegeding, 2012). This in turn affects the size, shape, and spatial arrangement of the aggregates that further impact the mechanical and microstructural properties of the resulting gels, and ultimately the texture of food products in which they are incorporated.

A decrease in heating temperature from 90 to 70°C results in lesser protein unfolding and fragile gels are formed, whereas at higher temperature and slower heating rates, a strong WP gel with enhanced water holding capacity is obtained (Resch et al., 2005). Additional heating causes more proteins to adsorb to the originally-formed network and thicken it. During cooling or low temperature storage, protein continues to polymerize, strengthen the gel and interact with additional water via hydrogen bonding. A relatively weaker and brittle WP gel is formed at acidic pH due to the increase in the denaturation temperature of the WP (Bryant & McClements, 2000a; Havea et al., 2009),

enhanced intermolecular repulsive electrostatic interactions and suppression of thiol group activity (Shimada & Cheftel, 1989).

Figure 2.2 illustrates how pH and ionic strength influence the gel properties during heat-induced gelation. Two types of WP protein gels are formed: fine-stranded and particulate gels. Heating a protein solution at pH values far from the isoelectric point of WP ($4 < pH > 6$), and at low ionic strengths, results in predominantly intermolecular electrostatic repulsion due to the highly charged protein molecules. Under these conditions protein molecules form smaller and linear aggregates attributed to slower and more specific aggregation. The resulting fine-stranded gel is optically transparent with a good water holding capacity. Shifting the pH towards isoelectric point or increasing ionic strength reduces intermolecular electrostatic repulsion and denatured proteins rapidly aggregate randomly. In this case, a turbid particulate gel with a lower water holding capacity is formed, attributable to large inter-particle pores. At intermediate salt concentrations, gel network consists of a mixture of particulate and fine-stranded protein aggregates (Bryant & McClements 1998; Langton & Hermansson, 1992). The effects of varying the concentration and type of salts on structural properties of heat-induced WP gels have been investigated by researchers (Khun & Foegeding, 1991). WPI suspensions heated at 90 °C for 15 min, without the addition of salt, only readily formed gels at a minimum of 14% protein, while in presence of 20 mM $CaCl_2$, a lower level of protein was needed to form a self-standing WPI gel (Zirbel & Kinsella, 1988).

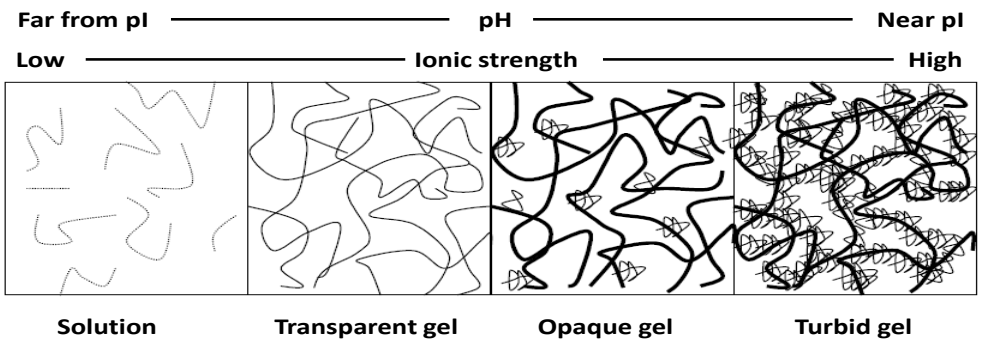

Figure 2.2. Relation between gel appearance and modulation of pH and ionic strength (Doi & Kitabatake, 1997)

2.6.2. Cold gelation of whey proteins

In many food applications, heating WP-containing products to high temperatures necessary for heat-induced gelation is undesirable. Therefore, formation of gel at room temperatures, termed cold-set gelation, is recognized to greatly extend the utility of WP. Cold gelation of pre-heated protein solutions has been reported for β-la, WPC, and WPI (Alting, Hamer, de Kruif, & Visschers, 2000; Barbut & Foegeding, 1993; Elofsson, Dejmek, Paulsson, & Burling, 1997; Sok Line et al., 2005). The process consists of two consecutive steps. In the first step, protein solution is partially or fully denatured, resulting in a significant degree of aggregation. Gelation is induced in the second step via reduction of electrostatic repulsion between the protein aggregates.

Preheating WP solution is the most commonly used method to produce 'reactive' WP aggregates. WP solution at neutral pH and containing protein concentration lower than the critical gelation concentration is usually heated between 68.5 to 90 °C for 30 min to 2 h to achieve a denaturation and aggregation degree of > 95%. Under these

conditions, the proteins will have a high net surface charge and predominantly electrostatic repulsive forces, resulting in the formation of disulfide-linked soluble aggregates that do not gel even after cooling. The aggregates are the building blocks of the final gel structures, in which they will assemble as larger aggregates. Many studies have demonstrated that large aggregates formed at pH 7 are connected predominantly through intermolecular disulfide bonds, with the presence of some degree of non-covalent interactions such as hydrogen and hydrophobic bonds (Alting et al., 2000; Alting, Hamer, de Kruif, Paques, & Visschers, 2003; McClements & Keogh, 1995; Sok Line et al., 2005). The effect of WP concentration during the preheating step at pH 7 was studied by Ju and Kilara (1998b). Increasing WPI concentration increased the aggregate size from 20 nm at 2 wt.% to 60 nm at 9 wt%. The microstructure of cold-set gel can be modified by controlling the pre-heating temperature because it determines the degree of protein unfolding and thus the degree of protein-protein interactions during cold gelation. Pre-heating of WP solution at 70 °C results in translucent cold-set gel compared to opaque gel at 90 °C. Gels with higher water holding capacity are obtained at higher pre-heating temperatures (Hongsprabhas & Barbut, 1996).

In the second step, gelation of the primary aggregates at room temperature is achieved by changing solvent quality via addition of salts (salt-induced gels) or reduction of pH (acid-induced gels) (Alting et al., 2004; Bryant & McClements, 2000b; Hongsprabhas & Barbut, 1997). Acid-induced cold-set gel is usually formed by using glucono delta lactone (GDL). In aqueous solutions, GDL will slowly hydrolyze to gluconic acid, causing a gradual lowering of the pH and screening the electrostatic repulsion among the protein aggregates (Alting et al., 2000, 2004). Acidification rate

controls the level of structural rearrangements during gel formation. Addition of GDL results in more regular gels whereas irregular gelation is obtained with inorganic or other organic acids such as ascorbic or citric acids due to instantaneous decrease of pH (de Kruif, 1997). However, gel hardness increases with increasing acidification rate. Mechanical properties of the gels are influenced by the final pH. Maximum gel strength is observed at pH ~ 5 that is close to the isoelectric point of β-lg whereas elastic modulus and stress at fracture decrease at lower pH (Cavallieri, Costa-Netto, Menossi, & Da Cunha, 2007; Cavallieri & de Cunha, 2008; Ju & Kilara, 1998c). In general, gels produced by acidification are stronger than salt-induced gels (Dissayanake et al., 2010; Ju & Kilara, 1998a) due to finer networks of the former.

Salts induce gelation in WP by screening electrostatic interactions among charged protein molecules as a result of charge neutralization, leading to a decrease in electrostatic repulsions and consequent protein aggregation (Bryant & McClements, 1998). At the same ionic strength, divalent salts such as calcium have stronger effects on gelation properties of preheated WP solution than monovalent salts such as sodium. Maximum gel hardness was observed at 200 mM NaCl that is about 10–20 times higher than with $CaCl_2$ required to obtain the same hardness (Bryant & McClements, 2000a; Ju & Kilara, 1998b; Mulvihill & Kinsella, 1988). The results were attributed to lower aggregation rates for sodium-induced gels than for calcium-induced gels. This is partly due to the ability of divalent ions to form salt bridges between the negatively charged carboxylic groups on neighboring WP molecules (Bryant & McClements, 1998, 2000b; Marangoni, Barbut, McGauley, Marcone, & Narine, 2000). An increase in the strength and opacity of cold-set WP gels was observed with increasing calcium levels (Clare,

Lillard, Ramsey, Amato, & Daubert, 2007; Mudgal, Daubert, Clare, & Foegeding, 2011), which then remains constant (Bryant & McClements, 2000b; Khun, Cavallieri, & Lopes da Cunha, 2010) at 12.3 to 40 mM or decreases (Hudson et al., 2000) with further increase (>30 mM) in the salt concentration. Addition of high levels of salt favors the formation of large aggregates through the association of smaller ones (Durand, Gimel, & Nicolai, 2002), which weakens the gel network.

Cold-set WP gels form fine-stranded microstructures whereas heat-induced gels exhibit a particulate structure at the same salt concentration and pH, attributed to the slow interactions of divalent or monovalent ions with the WP polymers (Barbut & Foegeding, 1993; Khun et al., 2010). Similarly, cold-set WP gel has higher WHC with a finer structure even at a high $CaCl_2$ concentration (360 mM), compared to the heat-set gels with 10 mM $CaCl_2$. A higher shear stress is obtained in the cold-set gel at a low $CaCl_2$ concentration (10 mM) (Barbut, 1995; Roff & Foegeding, 1996) in comparison to the heat-induced gel. A lower $CaCl_2$ level is required to induce cold gelation of WP in comparison to ~10 mM required for heat-induced gelation (Khun & Foegeding, 1991; Barbut & Foegeding, 1993).

Figure 2.3 compares the steps for heat-induced versus cold-set gelation process. In contrast to heat-induced gelation, in which aggregation and gelation are intertwined, the two processes can be studied separately in a cold gelation procedure. In the first step it is possible to control and manipulate the properties of the aggregates by different heating strategies or chemical treatments before heating (Elofsson et al., 1997; Hongsprabhas & Barbut, 1997; Ju & Kilara, 1998b). In the second step it is possible to study how the properties of the aggregates influence the gelation process.

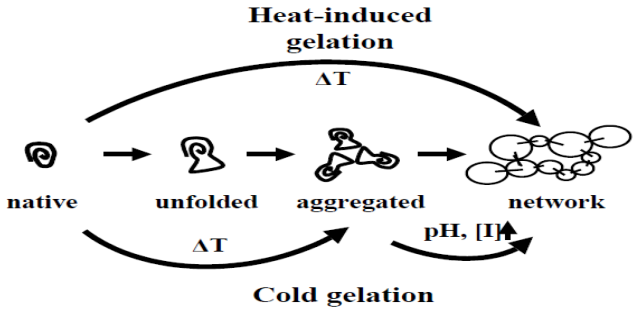

Figure 2.3. Conversion of native globular protein into a protein netwok following heat-induced gelation and cold-gelation procedures (Alting et al., 2003)

Most of the researche done to date have reported the cold-set gel formation from aggregated WP solutions. Only a few studies were performed on cold-gelling WP ingredient in dried form. Thomsen (1994) described a commercially available modified WPC powder produced by heat treatment during homogenization of WPC at slightly alkaline pH followed by drying. The modified WPC powder formed gel upon reconstitution in a salt solution. Hudson et al. (2000) and Resch, Daubert, and Foegeding (2004) developed a procedure for production of cold-set thickening WP ingredient without any addition of salt or heat. The derivitized WPC and WPI were obtained through a process consisting of pH adjustment to 3.35, thermal gelation at 80 °C, freeze drying and grinding to powder or spray drying the semi-solid gel. The derivatized proteins powders at 5-13% (w/w) imparted an instant thickening capability when reconstituted in water. The rheological characteristics of the derivitized powders are fairly independent of temperatures and pH (4 or 8) making them suitable for a wide range of food systems (Firebaugh & Daubert, 2005; Hudson & Daubert, 2002; Resch et al.,

2004). In the presence of supplemental calcium, the derivitized WPC showed increased thickening capacity especially under refrigeration temperatures (Clare et al., 2007). Dissayanake et al. (2010) established a method to produce a cold-set WP from microparticulated WP powders (MWP). MWP were produced from whey retentate standardized to 10% (w/w) protein followed by microfluidization at 140 MPa and heat treatment at 90 °C for 20 min. The heat-treated, microfluidized WP was spray dried to obtain the modified powder. The MWP powder at 12% (w/w) in water formed cold-set gels with the addition of GDL and/or NaCl (0.5 – 1.0 M). The gels had greater elastic properties compared to their heat-treated controls.

The cold gelation of WP has been demonstrated to have vast potentials in the food industry as an alternative for currently used polysaccharide-based thickening ingredients (Bryant & McClements, 1998). Therefore, controlling the polymerization of WP offers a new and interesting approach to modify and improve the physicochemical and nutritional properties of wide array of food products. Protein-based thickening agents could succeed in applications where it is desirable to reduce consumption of carbohydrates while increasing the consumption of proteins.

2.7. Emulsification of whey proteins

The amphiphilic nature of WP allows the protein to rapidly adsorb at the interface of fat droplets, followed by partial unfolding at the interface to form protective layer that prevents emulsion destabilization. The adsorbed protein forms intermolecular associations with adjacent protein molecules on the same droplet via hydrophobic,

electrostatic interactions and thiol/disulfide interchange reactions. The polymeric protein stabilizes emulsion primarily by a combination of steric and electrostatic interactions (Dickinson, 2001; Dickinson, Rolfe, & Dalgleish, 1990; Funtenberger, Dumay, & Cheftel, 1995). The effectiveness of proteins as emulsifier depends on the number and type of contacts they make with the interface. Protein molecule that is flexible and has a high proportion of hydrophobic residues is more effective at reducing interfacial tensions because of its ability to create more contacts with oil surfaces (Dickinson, 1999; Dickinson, Murray & Stainsby, 1988). Several treatments have been proposed to improve interfacial properties of WP including thermal treatment (Dissanayake & Vasiljevic, 2009; Zhu & Damodaran, 1994), high pressure processing (Desrumaux & Marcand, 2002), crosslinking via enzymatic reactions (Clare & Daubert, 2011; Eissa et al., 2004) and conjugation with carbohydrates (Akhtar & Dickinson, 2003).

The interfacial properties of WP are also directly linked to its solubility, size, charge and flexibility, which vary based on the environmental factors (pH, ionic strength) and modification processes. The effect of heat treatment on the structural and emulsifying properties of whey protein has been documented (Firebaugh & Daubert, 2005; Sok Line et al., 2005; Dissanayake & Vasiljevic, 2009). Conflicting results have been reported on the effects of heat treatment on the emulsifying properties of WP. Several workers have demonstrated that aggregation of protein impairs emulsifying properties (de la Fuente, Singh, & Hema, 2002; Millqvist-Fureby, Elofsson & Bergenstahl, 2001), while others (Firebaugh & Daubert, 2005) reported that emulsifying abilities of pre-heated WP remains the same as non-heated protein. In contrast, Britten et al. (2005) demonstrated that modification of protein via heat treatment improved the

emulsifying activity but not the emulsion stability. The improved emulsification was attributed to increased surface hydrophobicity of protein that enhanced its affinity to the oil-water interface, which also leads to the formation of thicker protein membranes (Britten et al. 2005; Dickinson, 1999; Mitidieri & Wagner, 2002). WPC-based emulsions made from a completely denatured WPC (preheated at 85°C for 15 min) were reported to have the lowest temperature cycling stability in terms of firmness and droplet size compared to WPC preheated at lower temperatures (Kiokias and Bot, 2005). Emulsions made from a composite of aggregated and native proteins demonstrate an improved emulsifying characteristics due to complementary roles of both native and aggregated proteins, in which the native WP rapidly adsorb at the surface whereas the denatured proteins produce a thick membrane at the interface (Fainerman *et al*., 2006; Millqvist-Fureby et al., 2001).

Ionic strength is considered as one of the important factors in determining the stability of protein-stabilized emulsions due to their influence on electrostatic interactions (Hunt & Dalgleish, 1994; Kulmyrzaev, Chanamai, & McClements, 2000). The association of calcium ions with the adsorbed β-lg results in aggregation of emulsion droplets (Agboola & Dalgleish, 1995). However, the influence of mineral ions on the stability of protein-stabilized emulsion depends on other solvent conditions such as pH, nature of ions, and salt levels (Kulmyrzaeva & Schubert, 2004; Ye & Singh, 2000). In the absence of $CaCl_2$, extensive droplet aggregation occurred around the isoelectic point of the WP ($4 < pH < 6$) because of their low charges. In the presence of > 3 mM $CaC1_2$, extensive droplet aggregation and creaming instability occurred at pH 7 (Kulmyrzaev et al., 2000). The binding of calcium ions to WP reduces electrostatic repulsions and

promotes the interactions of hydrophobic residues that may affect the adsorption behavior of protein at the interface (Baumy & Brule, 1988).

The presence of other component such as polysaccharides also affects the stability of emulsions. Lower emulsifying capacity was observed in emulsions containing 2-3% starch (Farrag, 2008; Herceg, Rezek, Lelas, Kresic, & Franetovic 2007). This was attributed to the non-adsorbing properties of the biopolymer and also impairment of protein propagation to the interface due to the large sizes of starch molecules (Cao, Dickinson, & Wedlock 1990; Kim, Decker, & McClements 2003).

2.8. Modification of whey proteins by extrusion process

Thermoplastic extrusion is the most commonly used method to texturize WP (Martinez-Serna & Villota, 1992). Extrusion cooking is a continuous thermomechanical process with multifunction operations. It is a high-temperature (above 140 °C), short-time process in which proteins are plasticized and denatured by a combination of pressure, temperature, and shear, resulting in molecular transformation and chemical reactions (Harper, 1989; Cheftel, Kitagawa, & Queguiner, 1992). It has been widely applied to dairy ingredients to improve functional properties and broaden the range of their food applications. Whey protein-based products with new textural characteristics have been developed to create expanded, porous products for snack foods and fibrous texturized structures for use as meat analogs (Onwulata, Konstance, Cooke, & Farrell, 2003; Walsh & Carpenter, 2003).

The final texture of extruded products depends on many factors including physicochemical parameters (feed formulation and extruder operating conditions) and configurational parameters (extruder screw and barrel configurations) (Cheftel et al., 1992; Harper, 1989; Onwulata et al., 2003). For example, screw configuration affects pressure profile along the barrel which in turn affects the physical state of the proteins and their interactions. The presence of other ingredients during extrusion such as polysaccharides generally enhances protein aggregation (Cheftel et al., 1992), while lipids decrease the shear effect and particle alignment (Noguchi, 1989).

Several authors (Hale, Carpenter, Walsh, 2002; Taylor & Walsh, 2002; Walsh & Carpenter, 2003) extruded WPC-corn starch blends (2:1 ratio) at different levels of calcium (0.5–1.7%, w/w) and pH (4.0–7.5) at 32-50% moisture and product temperature of 55, 90, 130 and 140 °C to develop texturized WP (TWP) that can be used as meat analogs. The TWP produced with the presence of calcium and at low pH range had higher water holding capacity due to enhancement of protein cross-linking at the later stages of extrusion.

Queguiner et al. (1992) have demonstrated a micro-coagulation method of WP using extrusion processing to produce semi-solid fat substitutes with a spread-like consistency and a smooth texture. This was achieved by extrusion of WPI (20% protein) at 77% moisture and a barrel temperature of 90–100 °C. Under the influence of shear forces, partial coagulation of β-lg resulted in aggregated particles of < 20 μm in size. Products obtained at pH 3.5–3.9 had optimal firmness, cohesiveness, and smoothness while at higher pH (4.5–6.8) more intermolecular disulfide exchange reactions occurred, resulting in products with grainy textures. This product was readily dispersible in water

and could be used in reduced-fat acid foods. However, it may not be usable at high levels in non-acid foods or food that require high temperature processing due to possible increase in particle size, thus resulting in coarser texture. Pre-treatment of WPI before heat-induced gelation by extrusion at controlled shear at 25 °C has been reported to result in increased gel strength due to enhanced protein-protein interactions (Ker & Toledo, 1992).

Onwulata at al. (2003, 2006) have investigated the use of low temperature extrusion (< 100 °C) at various pH to alter the functionality of WP. Varying extrusion temperatures allows a different degree of WP denaturation that might be useful for different products applications. WPC and WPI extruded at 35, 50, 75 and 100 °C with 38% moisture exhibited increased protein insolubility at pH 7 with increasing extrusion temperatures. The gel strength of the extrudate increased from 35 to 50 °C but diminished when the protein was extruded at 75 and 100 °C. Modification of the pH of water stream has been demonstrated to influence functional properties of extrudates. Extrusion of WPI at alkaline conditions (pH 11.5–12.4) increased the solubility and pasting properties of dried extrudate, while pasting properties were reduced at acidic conditions (pH 2.0-2.5). Extruded WPI under alkaline conditions had a stringy texture that could be used as meat analogs.

Despite the increased use of extrusion processing of WP to create and improve the desired functional properties in dairy protein-based foods, it is still difficult to predict structures, texture, or functionality of WP resulting from the extrusion process (Purwanti, van der Goot, Boom, & Vereijken, 2010). The available knowledge is insufficient on the effect of process parameters including extrusion temperature, moisture content of the feed

and screw speed. Little is known about the chemical reactions occurred during extrusion and their implications on structures, textures, and nutritional characteristics of extruded products (Abd El-Salam et al., 2009; Purwanti et al., 2010) due to the diverse molecular compositions of proteins and complex macromolecular interactions that could occur during the extrusion process.

2.8.1. Chemical-crosslinking of protein during extrusion

The combination of shear, temperature, and pressure during extrusion processing creates opportunities for structure formation of protein extrudates resulting from molecular transformation and chemical reactions (Ledward & Tester, 1994). Several authors have attempted to investigate the mechanisms of protein texturization during extrusion cooking (Chiang, 2007; Ledward & Tester, 1994). The increase in pressure and temperature due to both transfer of heat from the heated barrel and by viscous dissipation of motor energy lead to extensive protein denaturation while the shear force cause alignment of protein matrix towards the die (Camire, 1991; Cheftel et al., 1992; Li & Lee, 1996). Circular dichroism and intrinsic tryptophan fluorescence spectroscopic studies revealed considerable conformational changes occurring at secondary and tertiary structures of extruded WPI as a function of increasing extrusion temperatures (Qi & Onwulata, 2011). Proteins become insoluble and aggregate into a microscopic structure that involves alteration of both covalent and non-covalent interactions. The proteins then cross-link at the die end of the extruder to impart a network to the extrudate (Martinez-Serna & Villota, 1992).

It has been proposed that during the extrusion process, proteins possibly (1) randomly aggregate or orient into spherical molecules, or (2) aggregate as strands either randomly or oriented in the direction of flow (Ledward & Mitchell, 1988). In earlier works, it was reported that disulfide bonds were of negligible importance in the final structure of soy protein (SP) extrudate (Burgess & Stanley, 1976). The authors suggested that new peptide bonds that form in the severe conditions of extrusion (~ 180 °C) were responsible for the formation of new structures, as indicated by an increase in free sulfhydryl content and a decrease in disulfide bonds. However, the proposed mechanism was disputed. An almost complete solubilization of WP and SP extrudates in β-mercaptoethanol indicates that intermolecular disulfide bonds are the prevalent interactions that occurred during extrusion while hydrogen bonds, hydrophobic and electrostatic interactions had smaller roles (Arêas, 1992; Jeunink & Cheftel, 1979; Li & Lee 1996; Lin, Huff, & Hsieh, 2000). The authors reported that there is lack of evidence for significant formation of intermolecular peptide bonds and other covalent bonds. In a recent study, Chen, Wei, and Zhang (2011) demonstrated that non-covalent bonds surpass the importance of covalent bonds in SP extrudates obtained at 28 and 60% moisture content. However, the hydrophobic interactions, hydrogen and disulfide bonds were noted to collectively stabilize the structure of the extrudates. Similarly, Ledward and Tester (1994) proposed that linkages other than disulfide bonds were more important in initial structure formation of extrudates. The disulfide linkages were assumed to rupture at high temperatures (140–180 °C), but may have reformed only during cooling (Cheftel et al., 1992; Ledward & Tester, 1994).

The importance of electrostatic repulsions has also been explained as a function of change in the net charge of protein. The total charge of protein is proposed to likely increase as the protein melts travel along the barrel of the extruder due to pressure-induced ionization of groups such as carboxylate and amino groups of the protein. The repulsion between like charges creates more expanded and easily hydrated structures (Ledward & Mitchell, 1988; Ledward & Tester, 1994). During extrusion, protein aggregation from heating is balanced with deaggregation due to mechanical stresses (Redl, Guilbert, & Morel, 2003). High molecular weight proteins dissociate into smaller subunits (Areas, 1992; Camire, 1991), leading to an increase in their solubility (Mohammed, Hill, & Mitchell, 2000). Despite all the above proposed mechanisms, the precise changes in protein-protein interactions occurring within the extruder barrel are poorly understood.

2.8.2. Reactive supercritical fluid extrusion

Reactive extrusion is defined as the concurrent reaction during extrusion processingwherein an extruder is used as chemical reactor for polymerization and polymer modification (Michael & Grefenstein, 1995). It was developed in the 1980s, primarily for the modification of synthetic polymers and blends and later became important in food processing and starch modification for non-food applications (Xie, Yua, Liu, & Chen, 2006). The mechanisms of reactive extrusion can be combined in three main groups: rheology (flow), heat transfer, and reaction, in which they are interdependent on each other (Michael & Grefenstein, 1995). It involves processes such

as viscosity-breaking, grafting, crosslinking, coupling reactions, and polymerization that result in modification of chemical and functional properties of feed polymers. The modified polymers have enhanced characteristics such as thermal stability, mechanical strength, elongation and adhesive properties (Xie et al., 2006).

Reactive supercritical fluid extrusion (RSCFX) is a novel extrusion technology developed for production of highly expanded starch and patented by Rizvi and Mulvaney (1992). At supercritical conditions (7.38 MPa, 31°C), supercritical carbon dioxide (SC-CO_2) has both liquid-like and gas-like properties, which allow for high solubilizing capacity for non-polar materials like lipids and certain flavors and high diffusivity in biopolymer melts (Chen & Rizvi, 2006; Yu, Singh, Rizvi, & Zollweg, 1994). The process was developed to replace the conventional extrusion method of steam puffing that normally involves low in-barrel moisture content (15-20%, wet basis) and high temperatures (130-170 °C) and shear (Harper & Tribelhorn, 1992). These harsh conditions prevent utilization of heat-sensitive ingredients such as proteins, vitamins and certain flavors. In the RSCFX process, SC-CO_2 is utilized as a blowing agent, a nutrient carrier and as an in-line process modifier. Higher moisture content (20–60%) could be used to keep the product temperature low (<100 °C) via reduction of viscous dissipation of energy, which also help the solubility of SC-CO_2 in the melt. The diminution of viscosity results in limitation of mechanical stresses and a decrease in operating temperatures, allowing the process to be performed at low-shear conditions (Alavi & Rizvi, 2010; Rizvi, Mulavaney, & Sokhey, 1995).

The generic SCFX technology does offer the potential to provide a mechanism for unique textural and chemical modifications in various products. The potential of using

SCFX in producing a range of puffed food products such as ready-to-eat cereals, pasta and confectionery has been reviewed (Rizvi et al., 1995). In this process, expansion of extrudate is achieved by first solubilizing SC-CO_2 in the melt, and then inducing cell nucleation due to controlled pressure drop before the die which is followed by exit from the die where cell growth caused by diffusion of SC-CO_2 into the nucleated cells occurs (Alavi, Gogoi, Khan, Bowman, & Rizvi, 1999; Rizvi et al., 1995).

The SCFX process is more versatile and controllable. It allows for the manipulation of operating conditions such as in-barrel pressure, screw rotation speed, feed rate, moisture content, SC-CO_2 injection rate and product temperature at the die (Alavi & Rizvi, 2005). Therefore, microcellular structures of different morphologies such as cell size, cell density and expansion ratio can be varied to obtain expanded products with desired mechanical properties. The chemical environment in RSCFX can be manipulated by changing conditions of injected SC-CO_2 to attain different pH levels and alteration of ionic strength coupled with heat. This will result in alteration of the conformational structure of extruded products attributed to the exposure of reactive groups of amino acids, which eventually helped to modify the functionality of texturized protein (Manoi & Rizvi, 2008).

The effect of SC-CO_2 on the functionalities of WP has been documented (Xu, Yuan, Jiang, Wang, Hou, & Gao, 2011; Zhong & Jin, 2008). Enhanced gelation properties of WPC and WPI powders and dispersions treated with SC-CO_2 have been observed. The WPI was exposed to SC-CO_2 at 40 °C and 10 MPa for 1 h whereas WPC and WPC powders were treated at 65 °C for 1 h at 10-30 MPa (Zhong & Jin, 2008). The observed improvements in the gelling properties were attributed to the conformational

changes in protein structures leading to partial denaturation and exposure of reactive hydrophobic groups (Xu et al., 2011). These authors demonstrated changes in secondary structures as indicated by a decrease in α-helix content and hydrogen bonds and an increase in the amount of β-sheet.

Manoi and Rizvi (2008) established a new modification method of WPC using the RSCFX process. The process involved a WPC prehydration step, in which 6% wt. pregelatinized starch, 0.6% wt. NaCl and 0.6% wt. CaCl2 were blended together. The protein blend was extruded at a low temperature (90 °C) and at acidic and alkaline pH (pH 2.89–8.19) and 1% SC-CO_2 was injected into the system before the die exit. The resulting dried texturized WPC (TWPC) extruded at pH 2.89 formed a cold-setting gel upon reconstitution with water at 20% (w/w) TWPC, and was stable over a wide range of temperatures. The TWPC had excellent emulsifying properties compared to commercial WPC and capable of stabilizing the emulsions against coalescence at lower protein contents (Manoi & Rizvi, 2009).

2.9. References

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CHAPTER 3

FUNCTIONAL PROPERTIES OF WHEY PROTEIN CONCENTRATE TEXTURIZED AT ACIDIC pH: EFFECTS OF STARCH AND CALCIUM

3.1. Abstract

Whey protein concentrate (WPC) with and without added starch and with two levels of calcium was modified at pH 3.0 by reactive supercritical fluid extrusion (RSCFX). The texturized WPC (TWPC) exhibited 200- to 300-fold greater viscosities than non-texturized WPC at varying concentrations and formed cold-set gels at 20% (w/w) protein upon reconstitution in water. Emulsions containing 20% (w/w) oil prepared with 4% (w/w) TWPCs were very stable during 14 days of storage at room temperature compared to non-texturized WPC that became unsatble within 24 h of storage. The starch-containing sample (TWPC-S) was less soluble, consisted of larger aggregates (1.527 μm), showed lower emulsifying properties, but had higher apparent viscosity than TWPC alone due to synergistic contributions of the two biopolymers. TWPC produced without starch and with lower (0.3%) calcium level had smaller aggregates (0.996 μm) with higher protein solubility. The results provide a new approach by which functional characteristics of TWPC ingredients may be advantageously designed by altering the formulation composition.

3.2. Introduction

Whey protein (WP) is a multi-component mixture with high nutritive values. The unique functional characteristics of WP, such as hydration-related and surface-related properties, make it a useful food ingredient for a wide array of applications (Bryant & McClements, 1998; Morr & Ha, 1993). WP is able to thicken solutions and form heat-induced gels by a three-stage process involving unfolding, aggregation, and association of protein molecules via protein–protein and protein–water interactions. However, the protein needs to be heated to high temperatures (> 65 °C) for the process to occur (Livney, Corredig, & Dalgleish, 2003; Roefs & de Kruif, 1994), and this limits its applications in many food formulations. Therefore, the need for ingredients with novel functionality such as the induction of gelation at ambient temperatures provides the motivation to further modify WP into superior products.

A process for manufacturing cold-gelling proteins has been reported for β-lactoglobulin (β-lg), whey protein concentrate (WPC), and whey protein isolate (WPI). The process combines a controlled thermal denaturation of WP solution to form soluble aggregates, followed by a gelation step at low temperatures (20° to 40 °C) by reducing electrostatic repulsion between the protein aggregates via addition of salt or reduction of pH (Alting, de Jongh, Visschers, & Simons, 2002; Barbut & Foegeding, 1993; Cavallieri & da Cunha, 2008). The polymerized WP exhibits thickening and gelling capacity that is stable over a wide range of temperature and pH values (Hudson, Daubert, & Foegeding, 2000). The resulting ingredient provides the food industry with more alternatives in product formulations, including superior nutritional values when compared with

polysaccharide-based stabilizing agents such as pregelatinized starch, carrageenan, and xanthan gum (Hudson & Daubert, 2002; Resch, Daubert, & Foegeding, 2004).

Although the polymerized cold-gelling WP has been shown to act as a thickening ingredient, there have been only a few techniques proposed to produce the cold-set WP thickening ingredient in powder form. Hudson et al. (2000) and Resch et al. (2004) established processing protocols involving the formation of semi-solid, acidified, and heated WP gels followed by freeze or spray drying. A high thickening capacity was obtained upon reconstitution of the powders in water without the presence of acid or salts. Microparticulation of whey retentates (pH 3.0) by microfluidization followed by thermal denaturation at 90 °C for 30 min and spray drying was proposed by Dissanayake, Liyanaarachchi and Vasiljevic (2012). The combination of heat and high hydrodynamic pressure resulted in WP micro-aggregates that were able to form a comparatively strong, cold acid-set gel. Extrusion technology is another technique that has been applied to improve the functional properties of dairy-based products. The combination of shearing screws, temperature, and moisture may be manipulated to alter the globular structure of WP, resulting in new functionalities (Onwulata, Isobe, Tomasula & Cooke, 2006; Queguiner, Dumay, Salou-Cavalier & Cheftel, 1992). Although the WP-based, cold-set thickening agent appears to have a great promise in food applications, a higher concentration of the protein ingredient was required to achieve thickening or gelling capacity similar to the traditional hydrocolloids, such as carrageenan or starch (Hudson & Daubert, 2002; Resch et al., 2004). Also, some of the parameters like high heating temperatures used during the process are known to have detrimental effects on the

emulsifying and foaming properties of the final products (Dissanayake & Vasiljevic, 2009; Dissanayakee et al., 2012).

Manoi and Rizvi (2008) for the first time utilized a novel reactive supercritical fluid extrusion (RSCFX) process as a new approach to modify WPC. The protein concentrate was texturized in the presence of pre-gelatinized starch and salts (NaCl and $CaCl_2$) using a combination of low shear (180 rpm), low-heat (<100 °C), and supercritical carbon dioxide (SC-CO_2) at acidic and alkaline pH (pH 2.89–8.19). The texturized WPC (TWPC) extruded at pH 2.89 formed cold-set gel upon reconstitution in water at 20% (w/w) TWPC powder. However, the presence of polysaccharide such as starch may impair emulsion stability, due to the non-adsorbing nature of the biopolymer that could be responsible for depletion-flocculation, which increases viscosity of water phase, leading to fast serum separation (Cao, Dickinson & Wedlock, 1990; Dickinson, Ma & Povey, 1994). Moreover, the presence of salt has been reported to regulate the gel strength and emulsifying properties of WP. Monovalent and divalent ions are able to reduce electrostatic repulsions, causing the protein to aggregate (Bryant & McClements, 2000; Marangoni, Barbut, McGauley, Marcone & Narine, 2000). Increasing the concentration of sodium and calcium ions has been reported to enhance cold-set gel strength, which then decreases upon further addition of salts (e.g. >30 mM $CaCl_2$). The increased rate of WP aggregation and formation of larger protein aggregates have been associated with increasing calcium levels (0–80 mM) (Clare, Lillard, Ramsey, Amato & Daubert, 2007; Mudgal, Daubert & Foegeding, 2011; Schokker, Singh, Pinder & Creamer, 2000). The divalent salts have stronger effects on gelation rate and final gel strength of preheated WP solution than monovalent salts at the same ionic strength

because of their ability to act as bridges between negatively charged carboxylic groups on neighboring WP molecules (Bryant & McClements, 1998; Marangoni et al., 2000). On the other hand, the presence of $CaCl_2$ induced the aggregation of fat droplets, attributed to a reduction in electrostatic repulsion between the droplets (Kulmyrzaev, Chanamai & McClements, 2000). Therefore, in the present study, the effects of starch and calcium levels on the physicochemical properties of TWPC were investigated. It is hypothesized that manufacturing TWPC with starch will result in stronger cold-setting gels while better emulsifying properties are obtained without starch but with reduced levels of calcium.

3.3. Materials and Methods

3.3.1. Materials and feed formulations

Commercial WPC-80 (Lactalbumin-49320) was purchased from Leprino Foods Company (Denver, CO, USA). The composition of the WPC was 81.5% protein (dry basis), 5.5% fat, 6.5% lactose and less than 3.0% ash. Pre-gelatinized corn starch (Xpandr® 612) was purchased from Tate & Lyle Ingredients (Decatur, IL, USA). Hydrochloric acid (15%, w/w) (JT Baker, Mallinckrodt Baker Inc., NJ, USA) was used as a pH-adjusting agent.

Thirty-four kg of commercial WPC-80 was pre-hydrated to 10% moisture (wet basis) by spraying an appropriate amount of water on the WPC-80 powder while mixing using a SP130 San Cassiano mixer (Roddi d'Alba, Piemonte, Italy). Three protein-blend formulations were prepared by adding other ingredients to the pre-hydrated WPC-80: (1) 6% (w/w) pre-gelatinized corn starch, 0.6% NaCl and 0.6% $CaCl_2$, (2) 0.6% NaCl and

0.6% $CaCl_2$, and (3) 0.6% NaCl and 0.3% $CaCl_2$, and were labeled TWPC-S, TWPC-0.6, and TWPC-0.3, respectively. The samples were pre-conditioned overnight before feeding into the extruder.

3.3.2. Texturization of whey protein by RSCFX

TWPC was produced using a RSCFX extrusion process previously described by Manoi and Rizvi (2009). The pre-conditioned sample was extruded using a pilot-scale Wenger TX-52 Magnum co-rotating twin screw extruder (Wenger Manufacturing, Sabetha, KS, USA) coupled with a supercritical carbon dioxide (SC-CO_2) injection system. The extruder with L/D ratio of 28.5:1 is configured for the RSCFX process and SC-CO_2 is injected into the barrel through four valves located at L/D ratio of 24. The extrudate was forced through two die inserts with 1.2 mm diameter circular openings. The sample was extruded at 90 °C and the barrel zone temperatures were maintained as shown in Figure 3.1. The extruder was operated at 130 rpm at a feed rate of 35 kg/h. SC-CO_2 (1.5%, w/w of dry feed) was continuously injected at a pressure of 10-15 MPa into the protein dough. HCl solution of 15% (w/w) was injected into the mixing zone to obtain a pH of extrudate of about 3.0 and the extrusion was carried out at 60% moisture content. The extrudates were dried at 40 °C for approximately 16 h to achieve 5–6% moisture content. The dried products were finely ground to pass through a 1.0-mm sieve (Thomas-Wiley Mill model ED-5, Arthur H. Thomas Co., PA, USA), and stored in air-tight containers at room temperature.

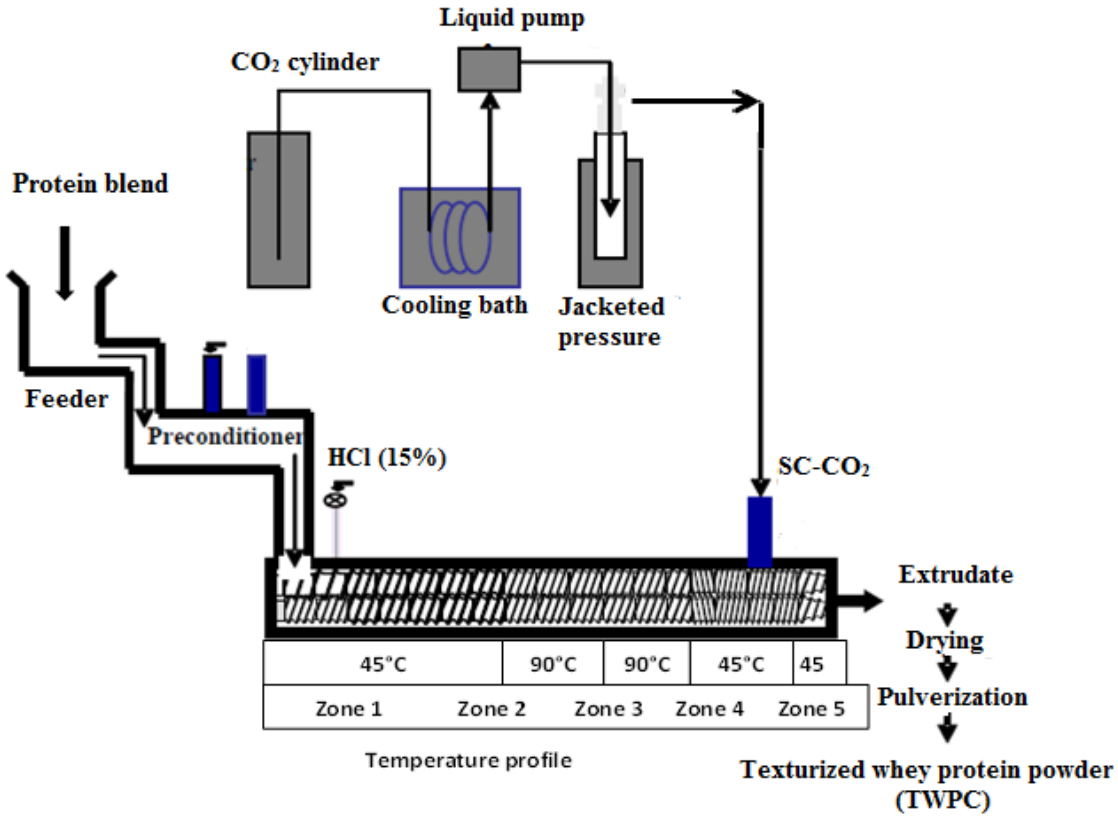

Figure 3.1. Schematic diagram of reactive supercritical fluid extrusion (RSCFX) system used for production of TWPC.

3.3.3. Particle size distribution

Particle size distribution of commercial WPC-80, non-texturized WPCs (pre-hydrated WPC with added starch and salts: WPC-S, WPC-0.3, WPC-0.6) and TWPCs (TWPC-S, TWPC-0.3, TWPC-0.6) dispersed in ultra-pure water (1.0%, w/w) was determined using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corp., Holtsviller, NY, USA). The samples were diluted to 0.10% to 0.05% (w/w) in ultra-pure water, immediately before the particle size measurements. Sample dilution was adjusted in order to achieve signal intensity of 400–800 kilocounts per second (kpcs). All measurements were performed at 20 °C at a fixed angle of 90° and a wavelength of 658 nm. Data was collected and analyzed using BIC software (Brookhaven Instruments Corp.). The intensity-weighed effective diameter (average diameter) and polydispersity index were determined for each sample. A polydispersity index close to zero (0.000 to 0.020) indicates monodispersed sample, a value in the range of 0.020 to 0.080 indicates narrow distribution, and a larger index indicates broader distribution.

3.3.4. Solubility determination

Protein solubility was determined according to the method of Shimada and Cheftel (1989) with minor modifications. Sample (1.0%, w/w, protein basis) was dispersed in deionized water and stirred at room temperature for 2 h. The sample was centrifuged at 20,000 g for 15 min at 20 °C. Total and soluble protein contents of the solution and supernatant, respectively, were determined using Bicinchioninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). The supernatant

and protein dispersions were diluted to 0.1% (w/w) with deionized water before measurements. Protein solubility was expressed as percentage ratio of protein contents in the supernatant and the dispersion before centrifugation.

3.3.5. Steady shear measurements

TWPCs and commercial WPC-80 powder were hydrated in deionized water to obtain a series of concentrations ranging from 4% to 20% (w/w). Non-texturized WPCs were not analyzed because preliminary data showed that the viscosities of the samples were not significantly different from the commercial WPC-80. Samples were stirred for 2 h and stored overnight at 4 °C for complete dissolution. Steady shear rheological measurements were performed at 25 °C using a strain-controlled rheometer (ARES, TA Instruments, DE, USA) equipped with a parallel plate geometry (50 mm diameter, 1.0 mm gap). The shear rate was ramped from 1.0 to 100 s^{-1}. Shear stress (τ) and apparent viscosity (η_a) were recorded by TA Orchestrator software. The flow curves were fitted to Power Law (equation 1) and Herschel-Bulkley (equation 2) models:

$$\tau = K\dot{\gamma}^n \quad (1)$$

$$\tau = \tau_0 + K(\dot{\gamma})^n \quad (2)$$

where K is consistency index ($Pa.s^n$), $\dot{\gamma}$ is shear rate (s^{-1}), n is flow behavior index, and τ_0 is yield stress (Pa).

3.3.6. Frequency sweep of cold-set TWPC gels

TWPC samples at 20% (w/w) in water were prepared as described in section 3.2.5. The control WPCs data is not included in the test because preliminary experimentation showed that the samples did not exhibit any viscoelastic behavior as observed from very low viscosity values. Frequency sweeps tests were performed for freshly prepared samples immediately after 2 h of stirring and after 24 h storage at 4 °C. Stored samples were equilibrated at room temperature for 1 h before the tests. The measurements were performed at 25 °C using a strain-controlled rheometer equipped with a cone and plate geometry (25 mm dia, 0.051 mm gap, 0.1° angle). Frequency was oscillated from 0.1 to 100 rad/s at 1.0 Pa strain within the linear viscoelastic region. Storage modulus (G'), loss modulus (G"), and loss tangent (tan δ) were recorded. The frequency dependence of G' and G" were described using a Power Law model:

$$G' = a\omega^{x} \qquad (3)$$

$$G'' = b\omega^{y} \qquad (4)$$

where ω is the frequency of oscillation (rad/s), exponents x and y represent the slopes of the relationship between modulus and frequency, and coefficients a and b represent the magnitudes of G' and G", respectively, at a given frequency.

3.3.7. Emulsifying activity index

Dispersions containing 1.0% (w/w) protein in 0.1 M phosphate buffer (pH 7.0) were prepared by stirring protein solutions for 2 h at room temperature. The samples

were stored overnight at 4 °C for complete dissolution. Emulsifying activity index (EAI) was determined by turbidimetric technique following the method of Pearce and Kinsella (1978) with some modifications. Emulsion was prepared by mixing 10 mL soybean oil and 40 mL protein dispersion using a high-speed dispersing unit, Ultra Turrax T25 basic (IKA Works, Inc., Wilmington, NC, USA), at 22,000 rpm for 2 min. The resulting emulsion (10 µL) was diluted in 5 ml 0.1 M phosphate buffer (pH 7.0) containing 0.1% sodium deodecyl sulfate (SDS) to suppress the flocculation of droplet. Absorbance was determined at 500 nm using a Spectronic 1200 spectrophotometer (Bausch and Lomb, Rochester, NY, USA). EAI (m^2/g) was calculated as:

$$T = (2.303 \times A) / l \quad (5)$$

$$\text{EAI } (m^2/g) = (2 \times T \times D) / (\varphi \times c \times 10000) \quad (6)$$

where, T is the turbidity, A is the absorbance at 500 nm, l is the path length of the cuvette (1 cm), D is the dilution factor, φ is the volumetric fraction of oil, c is the weight of protein per unit volume of aqueous phase before emulsification was formed (g/mL) and 10,000 is the conversion factor for square meters.

3.3.8. Creaming index

Emulsions were prepared as described in section 3.2.7. Each of the emulsions made with 1.0, 2.5 or 4.0% (w/w, protein basis) protein dispersions, resulting in final protein concentrations in emulsions of 0.8, 2.0 and 3.2% (w/w), respectively. The resulting emulsions (10 ml) were filled into glass test tubes (1.5 cm i.d. and 12 cm height) and then stored at room temperature. Height of the serum (H_s) and total height of

emulsion (H_t) were recorded periodically over a storage period of 1, 7 and 14 days. The stability of the emulsion was expressed as the Creaming Index.

Creaming Index (%) = (H_s / H_t) x 100 (7)

3.3.9. Scanning electron microscopy (SEM)

Microstructure of TWPC cold-set gel (20%, w/w) was determined using SEM. The gel was fixed in a 2.0% glutaraldehyde in 0.05 M sodium cacodylate buffer solution (pH 7.0) for 2 h at 4 °C, followed by three rinses (10 min each) in cacodylate buffer. Sample was then post-fixed for 1 h at 4 °C in 1% osmium tetraoxide, rinsed three times (10 min each) in 0.05 M cacodylate buffer and then dehydrated (at 4 °C) in a graded ethanol series (25, 50, 75, and 90%, v/v). Dehydration was continued in 100% (v/v) ethanol (two changes, 10 min each), followed by critical point drying. The dried gel was mounted on aluminum stub and coated with 10 nm of gold/palladium in a sputter coater. The SEM images were captured using a scanning electron microscope: Stereoscan 440 Leica operating at 5 kV. Images with a magnification of 10 000x were recorded.

3.3.10. Statistical analysis

Statistical analysis was performed using MINITAB (version 16). Differences between means ($p < 0.05$) were determined by analysis of variance using the general linear models and least square means procedure. All tests were done in triplicates, except for SEM, in which duplicate measurements were performed.

3.4. Results and Discussion

3.4.1. Particle size

Table 3.1 shows the effective particle sizes and polydispersity indices of TWPCs and their respective controls. The particle sizes of all control samples were not significantly different ($p < 0.05$), ranging from 0.332 to 0.344 μm. The polydispersity indices of control WPCs ranged from 0.163 to 0.195, implying all samples had narrow size distributions. The RSCFX process produced significantly larger protein aggregates with broader size distributions. The TWPC-S consisted of the largest particles with average size of 1.527 μm, while WPC extruded in the absence of starch and with lower $CaCl_2$ content (TWPC-0.3) showed the smallest average particle size (0.996 μm). The results indicated that the extrusion conditions applied during the modification process induced denaturation and aggregation in WP molecules. When protein molecules denature, the reactive groups such as hydrophobic and thiol groups are exposed, which in turn promotes intermolecular protein-protein interactions, facilitating the formation of aggregates (Spiegel, 1999; de la Fuente, Singh & Hema, 2002).

Table 3.1. Average particle sizes, polydispersity indices and solubility of non-texturized WPCs and TWPCs[1].

Samples[2]	Particle size (μm)	Polydispersity index	Protein solubility (%)
WPC-0.3	0.341 ± 0.006 [d]	0.194 ± 0.014 [bc]	96.74 ± 2.95 [a]
WPC-0.6	0.344 ± 0.004 [d]	0.195 ± 0.014 [b]	93.56 ± 3.23 [ab]
WPC-S	0.332 ± 0.003 [d]	0.163 ± 0.027 [d]	93.15 ± 5.04 [ab]
WPC-80	0.339 ± 0.005 [d]	0.164 ± 0.021 [cd]	88.78 ± 4.59 [b]
TWPC-0.3	0.996 ±0.061 [c]	0.351 ± 0.029 [a]	33.55 ± 1.73 [c]
TWPC-0.6	1.218 ± 0.126 [b]	0.365 ± 0.018 [a]	31.92 ± 2.81 [c]
TWPC-S	1.527 ± 0.163 [a]	0.373 ± 0.036 [a]	29.51 ± 3.13 [c]

[1] Means with the same superscript within a column are not significantly different ($p < 0.05$). Values are the means of three replications.

[2] WPC-0.3 is non-texturized WPC-80 containing 0.3% $CaCl_2$ and 0.6% NaCl; WPC-0.6 is non-texturized WPC-80 containing 0.6% $CaCl_2$ and 0.6% NaCl; WPC-S is non-texturized WPC-80 containing 6% starch, 0.6% $CaCl_2$ and 0.6% NaCl; WPC-80 is commercial WPC-80. TWPC-0.3, TWPC-0.6 and TWPC-S contain the same formulations, except the samples were texturized.

The difference in particle sizes observed between TWPC-0.6 and TWPC-0.3 could explain the role of calcium in the formation of protein aggregates. The higher $CaCl_2$ level (33 mM) possibly led to a greater degree of protein aggregation in TWPC-0.6 compared to TWPC-0.3, which contained a lower level of $CaCl_2$ (16.5 mM). Similarly, it has been demonstrated by others (Britten & Giroux, 2001; Mudgal et al., 2011) that increasing the ionic strength of heated WP dispersions facilitates the formation of larger aggregates due to excessive interactions of β-lg monomers and dimers, which lead to polymer formation. Mudgal et al. (2011) reported the formation of flexible fibrils in the presence of 25 mM $CaCl_2$, whereas large aggregates with more branching were observed in heated β-lg solution containing 50 mM $CaCl_2$. This was attributed to the ability of calcium ions to form ionic bridges between the adjacent, exposed aspartic and glutamic acids on neighboring protein molecules (Kinsella & Whitehead, 1989; Marangoni et al., 2000; Pappas & Rothwell, 1991). In addition, the lowering of electrostatic repulsion facilitated protein aggregation via hydrophobic interactions (Britten & Giroux, 2001; Harwalker & Kalab, 1985; Marrangoni et al., 2000).

The larger protein aggregates in TWPC-S compared to those observed in TWPC-0.3 and TWPC-0.6 may possibly be attributed to the presence of relatively larger starch particles or greater crosslinking of the macromolecules during extrusion. Another study proposed that greater protein-protein aggregation occurred during the extrusion of mixed WPC-pregelatinized waxy corn starch (Allen, Carpenter, & Walsh, 2007). The protein and starch phase separated into two different regions, in which accelerated β-lg aggregation occurred in the protein-rich phase, thus leading to the formation of large aggregates in the system (Olsson, Stading, & Hermansson, 2000, 2002).

3.4.2. Protein solubility

The solubility of TWPCs and their respective controls in water is shown in Table 3.1. The control samples exhibited higher solubility, ranging from 88.8% to 96.7%, implying that water-accessible surfaces of the proteins in their native state were highly hydrophilic, thus promoting protein–water interactions (Zhu & Damodaran, 1994). The higher solubility of non-texturized WPCs compared to the commercial sample may possibly be attributed to the presence of salts. The calcium ions bind water molecules and facilitate hydrogen bonding between water and protein molecules (Clare et al., 2007; Turgeon, Gauthier & Paquin, 1992). On the other hand, a significant ($p < 0.05$) reduction in protein solubility was observed in all TWPC samples compared to their respective controls. The solubility of the texturized samples ranged from 29.5% to 33.6%; with the highest value for TWPC-0.3 and the lowest for TWPC-S. The reduced solubility values were in agreement with other reports on extruded, heat-treated and/or acidified proteins (Britten & Giroux, 2001; Onwulata, Konstance, Cooke & Farrell, 2003; Turgeon et al., 1992; Zhu & Damodaran, 1994). The loss of protein solubility in texturized samples has been attributed to the formation of insoluble aggregates due to heating, as indicated by the increased particle size.

The RSCFX modified proteins showed higher solubility than those previously reported for extruded WP. High insolubility (77.7 - 87.2%) has been reported for WPC and WPI extruded at 75 and 100˚C with 38% moisture content (Onwulata et al., 2003), corresponding to 22.3 and 12.6% soluble proteins, respectively. In a more recent study, Qi and Onwulata (2011) demonstrated that WPC extruded at 100°C with 50% moisture had only 3% soluble protein. The greater solubility obtained in the present study could

be due to the use of milder process conditions (low screw speed, high moisture) and the presence of SC-CO_2 that also acts as a plasticizer. The acidic condition during the RSCFX process also helps stabilize the WP against thermal denaturation due to extra internal hydrogen bonding and/or loss of localized attractive electrostatic interactions (Kella & Kinsella, 1988). In addition, SC-CO_2 dissolved in the acquous part of the polymer melt dissociates into carbonic acid and may thus further decrease the pH.

3.4.3. Steady shear measurements

Consistency coefficient (*K*), flow behavior index (*n*) and yield stress (τ_0) of the texturized and commercial WPCs at various concentrations are summarized in Table 3.2. The flow behaviors of the samples were well represented by both the Power Law and Herschel-Bulkley models as observed by their high regression coefficient (R^2) values of 0.825–1.000. The TWPC samples exhibited significantly greater viscosities ($p < 0.05$) than WPC-80 at any concentration tested.

Table 3.2. Power law parameters[a] of WPC-80 and TWPC dispersions or gels at various protein concentrations.

Sample	Parameters	WPC-80 / TWPC concentrations (%, w/w)						
		4%	6%	8%	10%	12%	14%	20%
WPC-80	K	0.003	0.006	0.006	0.009	0.011	0.015	0.018
	n	1.008	0.981	0.838	0.782	0.789	0.758	0.874
	τ_{HB}	0.001	0.002	0.002	0.003	0.005	0.007	0.009
TWPC-0.3	K	0.004	0.014	0.070	0.723	3.640	10.507	68.61
	n	0.951	0.748	0.693	0.327	0.167	0.124	0.029
	τ_{HB}	0.001	0.004	0.006	0.347	3.190	10.164	69.356
TWPC-0.6	K	0.003	0.008	0.092	0.542	2.840	8.125	59.68
	n	0.992	0.839	0.637	0.405	0.207	0.388	0.041
	τ_{HB}	0.001	0.003	0.013	0.255	2.296	7.351	60.890
TWPC-S	K	0.005	0.014	0.102	0.629	5.047	11.692	83.18
	n	0.878	0.742	0.668	0.412	0.277	0.130	0.020
	τ_{HB}	0.005	0.006	0.022	0.407	4.744	11.376	85.687

[a] Values are the means of three replications.

K: consistency index (Pa.s^n); n: flow behavior index derived from Power Law model; τ_{HB}: yield stress (Pa).

Stdv. range for K: 0.001 – 7.149; Stdv. range for n: 0.012 – 0.280; Stdv. range for τ_{HB}: 0.001 – 6.817.

Figure 3.2 shows the viscosity-shear rate profiles of the samples. The WPC-80 solutions behaved as Newtonian liquids at 4% and 6% proteins and formed shear thinning liquids at higher concentrations (8 to 20%), as indicated by the decreasing flow behavior indices (n) with concentrations. On the other hand, TWPC samples exhibited similar flow profiles, each had a characteristic of Newtonian liquid at low protein concentration (4%), which then progressed to form shear thinning liquids as the concentration was increased to 6–14%. A highly homogenous, weak cold-set gel-like texture was obtained at 20% (w/w) concentration of TWPC. At higher concentrations, it was likely that denatured proteins were packed closely together, promoting stronger protein molecular entanglements that eventually contributed to the viscosity increases (Rector, 1992). At 20% (w/w) protein concentration, the apparent viscosities of the TWPCs were enhanced by 200 to 300-fold compared to WPC-80 (Figure 3.3), with flow behavior index values of 0.02–0.04, in comparison with a high flow index (n = 0.87) of WPC-80. The apparent viscosity data revealed that TWPC-S exhibited 1.2- to 1.4-fold greater viscosity compared to TWPC-0.3 and TWPC-0.6.

The strongest cold-set gel (20%, w/w) was obtained with TWPC-S, while TWPC-0.6 formed the weakest network. Similarly, a cold-set thickening behavior was obtained for derivitized WPC (dWPC) and WPC (dWPI) produced by heating acidified WP solutions (pH 3.35) at 80 °C for 1 h which exhibited cold-set thickening behavior upon reconstitution in water (Hudson et al., 2000; Resch et al., 2004). In an earlier study, a semisolid spread was obtained when 20% WPI was extruded at ~pH 3.5 with 77% moisture and barrel temperature of 90–100 °C (Queguiner et al., 1992). The increase in yield stress (τ_{HB}) of the TWPC dispersions and gels was noticeable when the

concentration of the TWPC was increased to 12% (w/w). The cold-set TWPC gels at 20% (w/w) exhibited the highest yield stress values ranging from 60.89 to 85.69 Pa. The control samples did not exhibit any yield stress. A higher yield stress value represents a stronger network, thus a greater stress is required to initiate product flow.

The high apparent viscosity of TWPC was a result of the changes in protein conformation that increased molecular hydrodynamic volume and exposure of the reactive groups which enhanced intermolecular protein-protein interactions. The combined intermolecular volume and aggregate size have been recognized to increase dispersion viscosity via protein-water interactions due to hydrogen bonding between amino acid groups and water molecules (Foegeding, Li, & Bottcher, 1998; Kinsella & Whitehead, 1989). These interactions cause swelling and increase the radii of protein molecules, resulting in enhanced viscosity of the system (Clark, 1998; Schmidt, Packard & Morris, 1984). The use of SC-CO_2 has also been implicated in the increase of the thickening capacity of TWPC due to changes in conformational structures of proteins (Xu et al., 2011).

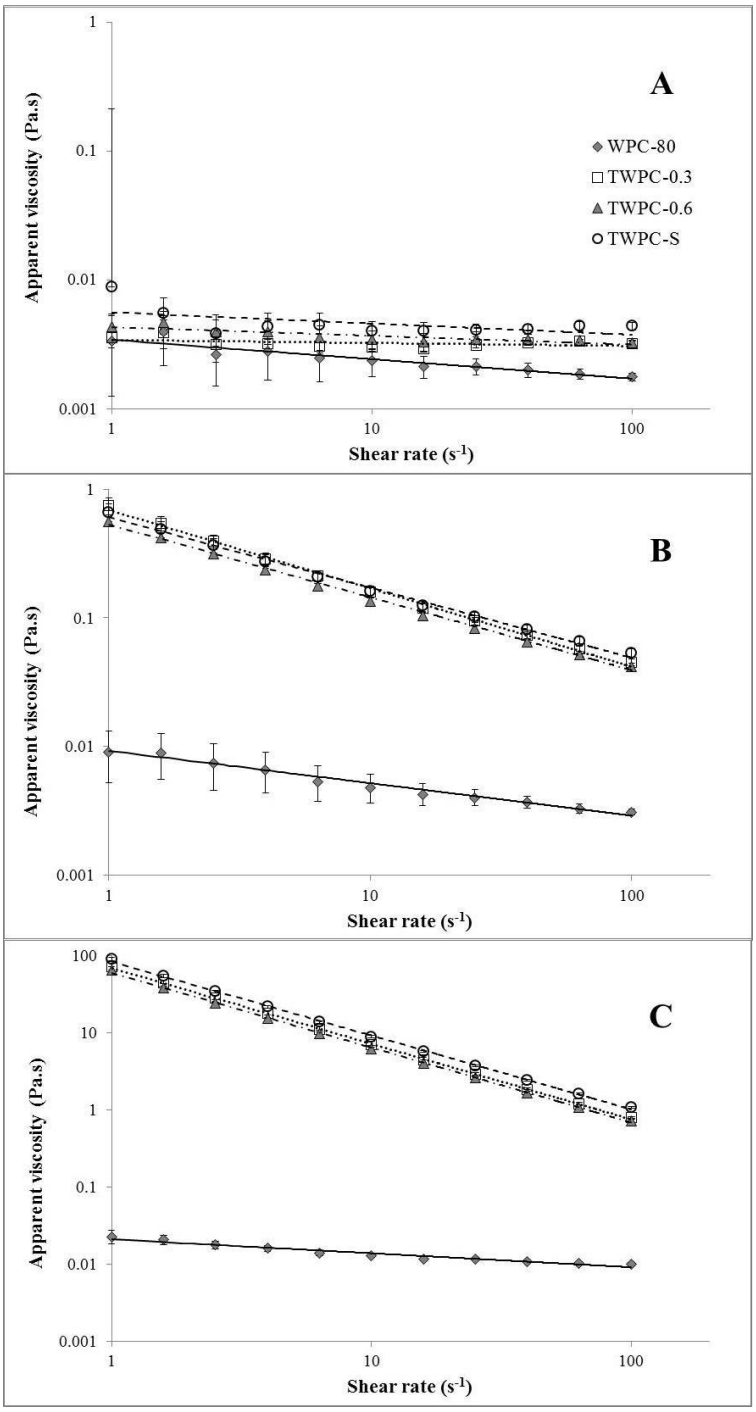

Figure 3.2. Effect of concentrations on flow behavior of WPC-80 (♦), TWPC-0.3 (□), TWPC-0.6 (▲), and TWPC-S (○) dispersions and gels. Samples were analyzed at (A) 4%, (B) 10%, and (C) 20% (w/w) WPC or TWPC.

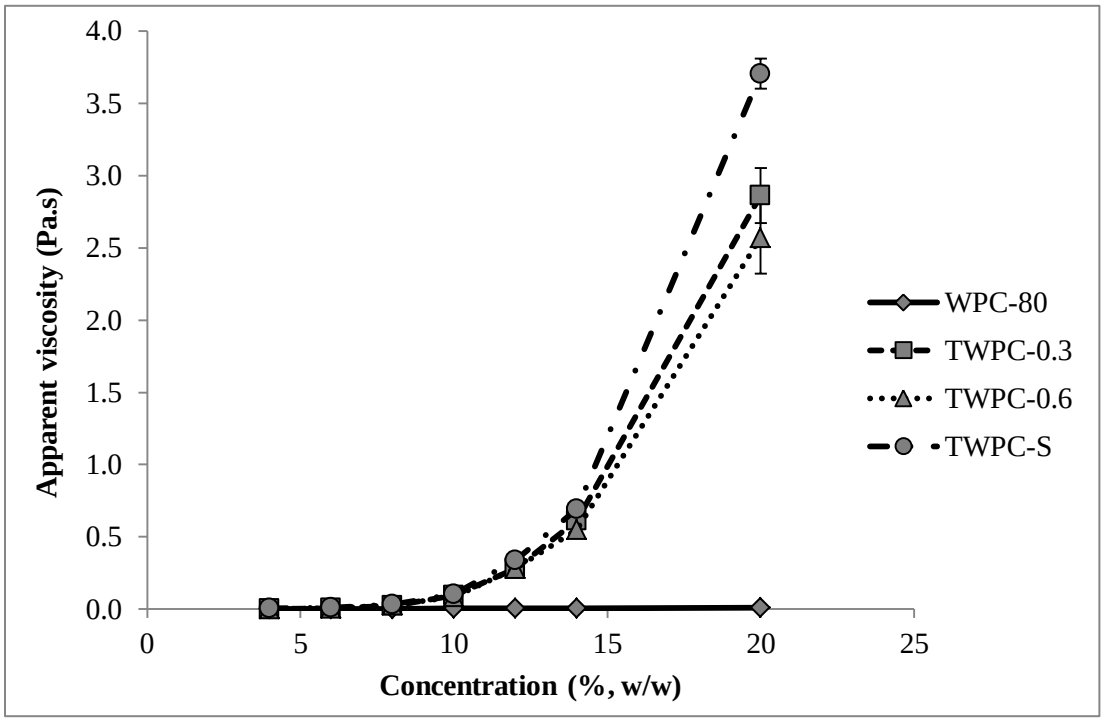

Figure 3.3. Variations in apparent viscosities (at 25 s^{-1} shear rate) of WPC-80 and TWPCs as a function of protein concentration.

The significantly enhanced viscosity of TWPC-S revealed that the protein and starch molecules were structurally compatible. The starch molecules perhaps embedded in the continuous protein matrix, thus synergistically reinforced the viscosity of the system. Several authors (Aguilera & Rojas, 1996; Lopes, Alviano, Torres, Gonçalves, & Andrade, 2006) have previously obtained stronger WP-starch mixed gels in comparison to gels made by a single polymer alone. The starch polymers acted as active filler in the continuous matrix of WPI gels due to their ability to form hydrogen bonds with water (Lupano & Gonzalez, 1999; Shim & Mulvaney, 2001). In addition, the starches were able to bind water to a greater extent than the protein molecules (Allen et al., 2007). It is interesting to note that in the present study, a significantly higher viscosity was obtained even at low starch weight fraction of 0.06, in comparison with high starch volume fraction (≥ 0.4) reported by Aguilera & Rojas (1996).

In the present study, it was demonstrated that increasing $CaCl_2$ to 0.6% slightly reduced the thickening capacity of TWPC compared with the system containing 0.3% $CaCl_2$. As previously discussed TWPC-0.6 experienced greater degree of aggregation and consisted of larger protein aggregates. The increment in calcium level was reported to increase the gel strength of heat-induced WP (Clare et al., 2007; Hongsprabhas & Barbut, 1996; Ju & Kilara, 1998), however, further increase in salt concentration (> 30 mM) resulted in weaker gels (Hudson et al., 2000). This observed behavior was suggested to be a function of larger protein aggregates resulting from calcium binding. The presence of a large portion of insoluble aggregates formed during polymerization of WP was reported to weaken the gel matrix, probably due the aggregates that acted as passive fillers (Britten & Giroux, 2001; Mudgal et al., 2011). From the present results, it

can be concluded that texturized WPC made with varying formulations show the ability to impart a wide range of viscosities at varying concentrations. It is possible to produce cold-set TWPC gels with high gel strengths with controlled additions of starch and $CaCl_2$.

3.4.4. Frequency sweep

The frequency dependence of G' and G" of freshly prepared (0 h) and aged (24 h) cold-set TWPC gels are shown in Figure 3.4. In all the samples, G' was higher than G", and both moduli showed slight frequency dependence as indicated by the low values of exponent *x* and *y*, ranging from 0.088 – 0.109 and 0.090 – 0.177, respectively (Table 3.3), implying a solid-like behavior. The exponent *x* is an indicator of the viscoelastic nature of the gels, in which a value near zero or zero implies a purely elastic or fully cured gel (Ikeda & Foegeding, 1999). A relative change of tan δ of freshly prepared samples over the entire range of frequencies is shown in Figure 3.5. The tan δ did not show much variations over the frequency range tested and varied from 0.127 to 0.286. The pattern of response as a function of frequency change was similar in all the fresh and stored samples. The values of tan δ were greater than 0.1, implying that the samples can be characterized as weak gels or pseudo gels with paste-like structure. The freshly prepared TWPC-S had the strongest gel network as indicated by the highest G' (314 Pa) and lowest exponent *x* and tan δ (0.129), while both of the TWPCs without the starch exhibited lower G' (157-196 Pa) and higher tan δ (0.147-0.148).

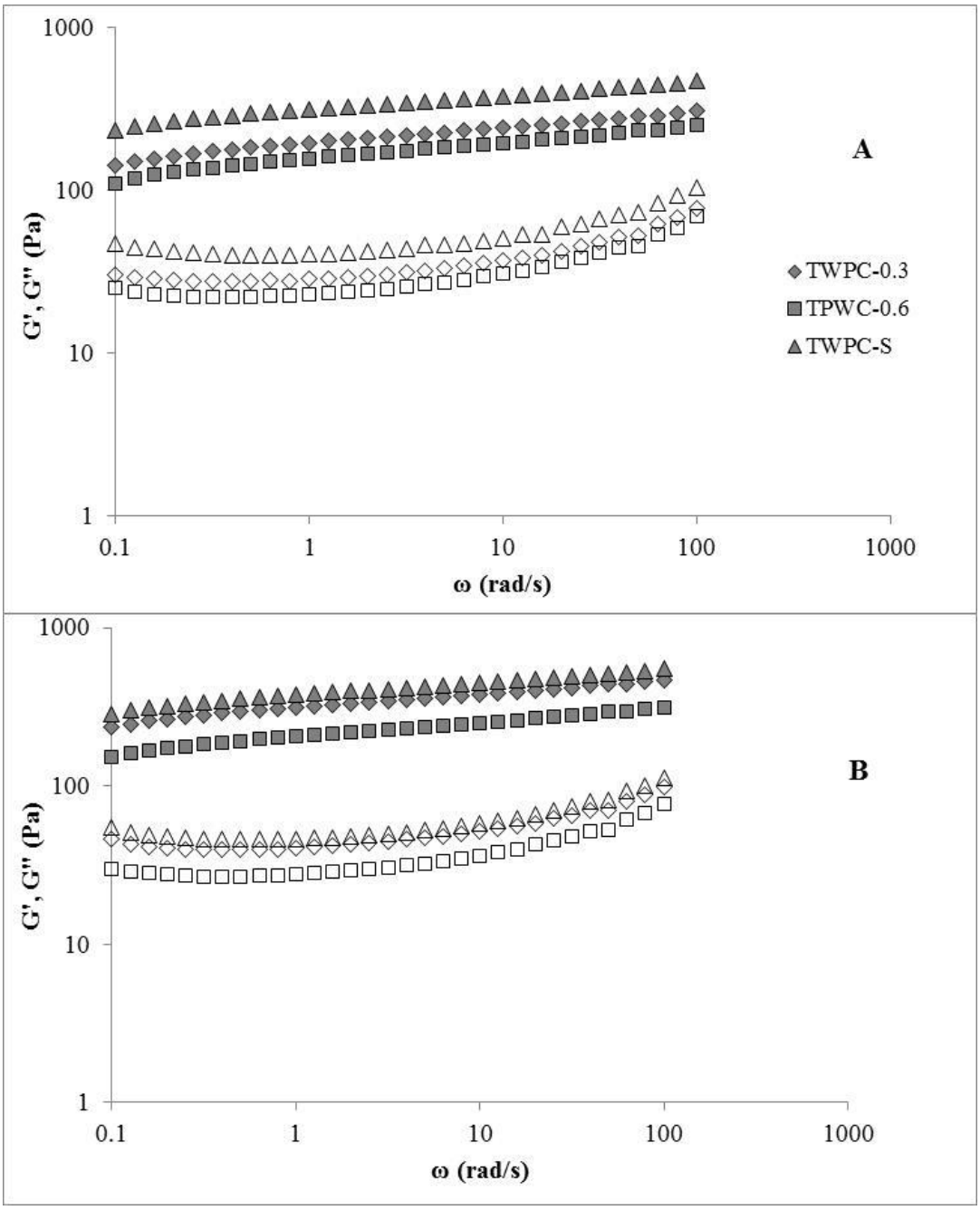

Figure 3.4. Variation of elastic (G') and viscous (G") moduli of cold-set TWPC gels at (A) 0 h and, (B) following 24-hour storage at 4 °C, as a function of frequency. Filled and empty symbols represent G' and G", respectively.

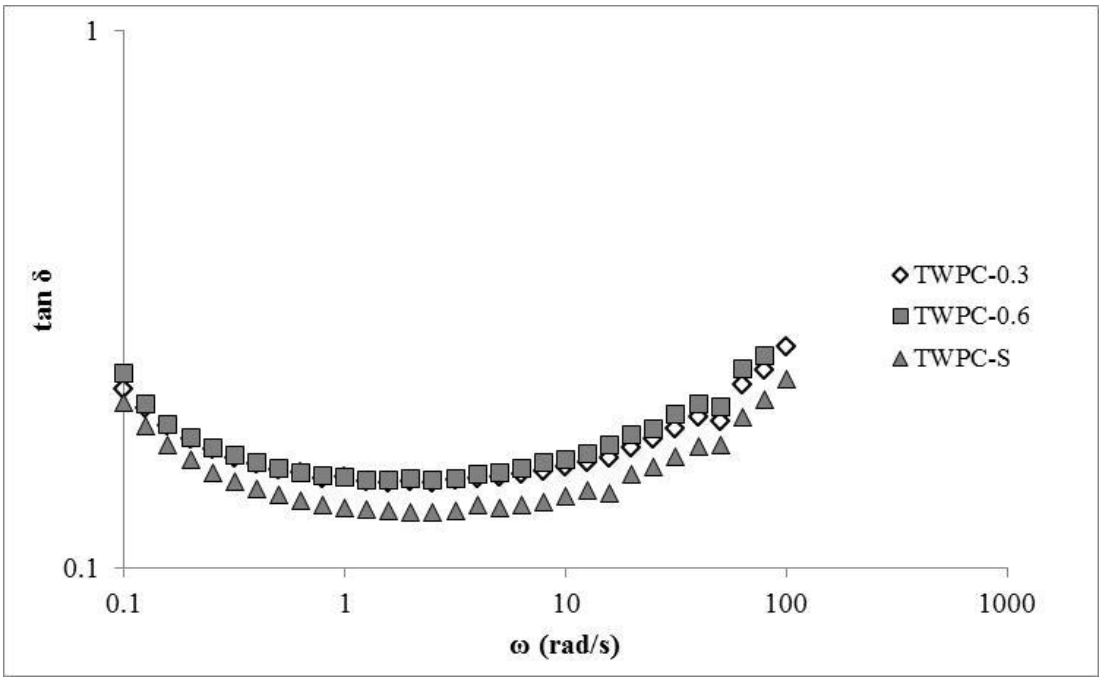

Figure 3.5. Variation of tan δ with frequency for freshly prepared (0 h) TWPC cold-set gels. Data for samples at 24 h not shown as they were similar in behavior.

The exponent *x* of all the samples decreased to 0.088 – 0.096 following storage for 24 h, implying that G' is less dependent on frequency. Concurrently, a significant increase in the moduli and a decrease in tan δ were observed, suggesting enhancement of the elastic properties of the gel network. The order of strength of the network formed at 24 h was similar to those of freshly prepared samples. The TWPC-0.3 samples showed the most pronounced increase (1.60-fold) in G' over the storage period, while TWPC-S showed the least increase (1.19-fold). Similar observations were reported by Clare *et al.* (2007) for cold-set WPC gels (pH 3.5) after 14 h storage at 4 °C, indicative of increased thickening at colder temperature settings. Aging of the cold-set TWPC gels may have

been due to the molecular rearrangement within the structure that consequently increases the protein–protein interactions via hydrogen bonds and hydrophobic interactions (Chou & Morr, 1979; Lanier, 2000; Renard, de Velde & Visschers, 2006). The increase in the amount and strength of intermolecular bonds decreases the pore sizes of the gels (van Camp *et al*., 1997; Johnston, Austin & Murphy, 1993), resulting in stronger matrices.

In comparison to TWPC-S, the higher protein contents of TWPC-0.3 and TWPC-0.6 possibly led to greater protein-protein interactions during aging of the cold-set gels. It is also assumed that the presence of pre-gelatinized waxy starch in TWPC-S did not contribute to the enhancement of the gel network, due to the lack of retrogradation attributed to the extensively branched amylopectin molecules that cannot readily align themselves with each other during ageing (Kim & Lee, 1987).

Table 3.3. Comparison of storage modulus (G'), loss modulus (G") and tan δ at 1 rad/s frequency (ω), and frequency dependence of G' and G" of fresh and stored cold-set TWPC gels[1].

Samples	Comparison at 1 rad/s			Power Law parameters[2]	
	G' (Pa)	G" (Pa)	tan δ	*x*	*y*
0 h					
TWPX-0.3	195.6^{c}	28.60^{b}	0.147^{a}	0.104^{b}	0.118^{a}
TWPC-0.6	156.9^{c}	23.08^{b}	0.148^{a}	0.109^{a}	0.130^{a}
TWPC-S	314.0^{b}	40.38^{a}	0.129b^{c}	0.092^{c}	0.102^{a}
24 h					
TWPX-0.3	312.2^{b}	40.55^{a}	0.130b^{c}	0.092^{cd}	0.177^{a}
TWPC-0.6	205.4^{c}	27.58^{b}	0.134^{c}	0.096^{c}	0.120^{a}
TWPC-S	375.2^{a}	46.14^{a}	0.124^{b}	0.088^{c}	0.090^{a}

[1] Means with the same superscript within a column are not significantly different ($p < 0.05$). Values are means of three replicates.

[2] *x* and *y* are exponents derived from Power Law model relating G' and G', respectively.

3.4.5. Emulsifying Activity Index

The emulsifying activity index (EAI), which represents the ability of proteins to adsorb at the interface of fat globules, is related to the surface area stabilized by a unit weight of proteins (Pearce & Kinsella, 1978). Experimental data revealed that the EAIs of non-texturized WPCs with additives were significantly ($p < 0.05$) higher (37.7 to 42.1 m^2/g) than commercial WPC-80 (35.7 m^2/g) (Figure 3.6). On the other hand, a slight reduction (5.6 – 15.8%) in EAI was observed in the texturized WPC, with values ranging from 35.6 – 37.6 m^2/g, in which TWPC-0.3 and TWPC-S had the lowest and highest reduction, respectively. In addition, no significant differences were observed between the TWPCs and commercial WPC. The presence of added salts in the non-texturized WPC might induce protein unfolding, thus exposing hydrophobic regions that further promote effective protein–lipid interactions at the oil–water interface via hydrophobic interactions. Inconsistent results have been reported for the emulsifying properties of heat-treated WPs. Several researchers demonstrated a decrease in emulsifying properties of WP dispersions when heat treated at either acidic or neutral conditions (Dissanayake et al., 2012; Voutsinas, Cheung & Nakai, 1983), whereas other authors found the emulsifying characteristics of heat-treated WP to be similar to their respective controls (Firebaugh & Daubert, 2005; Liu & Tang, 2011; Turgeon et al., 1992).

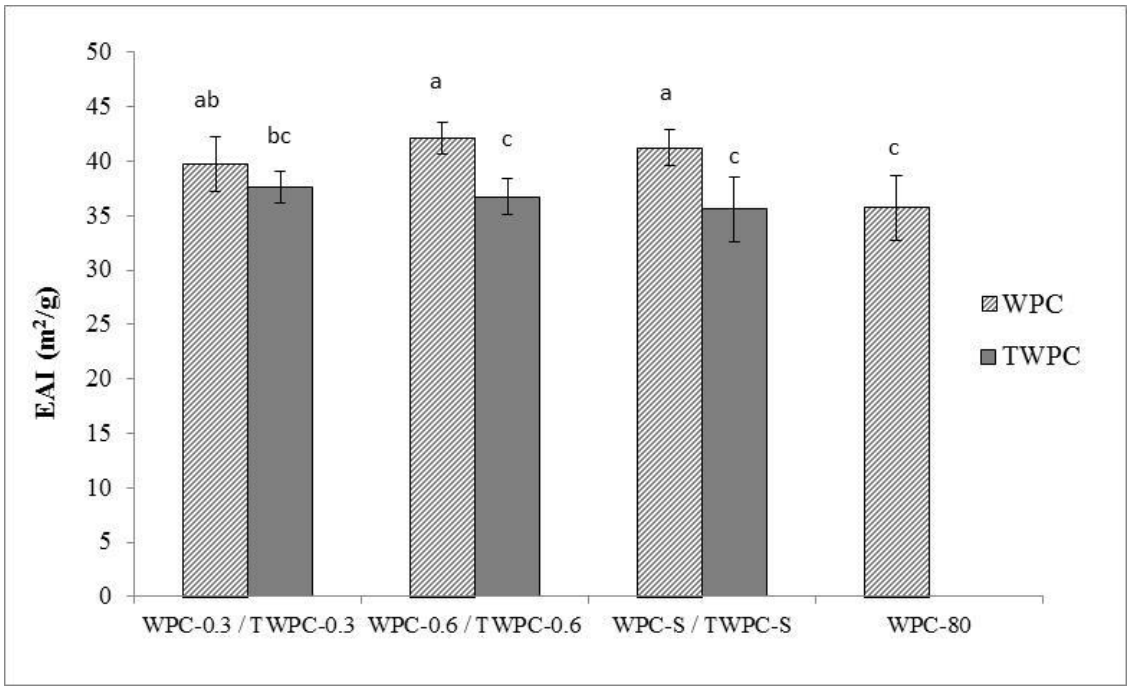

Figure 3.6. Emulsifying activity index of WPC and TWPC stabilized emulsions. Samples with the same superscript are not significantly different ($p < 0.05$). Bars show the standard deviation of three different measurements.

A number of factors were proposed to regulate the EAI of proteins. Native WPs were reported to have higher diffusivity towards oil–water interface, while reduced affinity was reported for aggregated proteins (Nicorescu et al., 2008), consequently reducing the availability of proteins to form and stabilize emulsions. However, considering that the EAI of TWPC was not significantly different from that of commercial WPC, other factors should also be involved in regulating the emulsifying capacity of TWPC. The higher emulsifying capacity of heated acidic WPC (pH 2.5) compared with native control was proposed to be due to the presence of more flexible

proteins in the former (Turgeon et al., 1992), which has been attributed to the formation of molecules with various degrees of unfolding (Harwalkar & Modler, 1981). It is assumed that the proteins in TWPC were in unfolded state with a greater number of exposed hydrophobic residueswhich gave the protein molecules better flexibility and affinity to form hydrophobic interactions at the oil–water interface. The presence of starch as a component of TWPC-S slightly lowered the EAI, due to the non-adsorbing properties of the starch polymers. In addition to little surface activity (Phillips & Williams, 1995), the large size of starch polymers possibly impeded protein migration to the interface (Kim, Decker & McClements, 2003).

3.4.6. Creaming Index

The creaming index (CI) provides an indirect indication of the susceptibility and degree of droplet destabilization via flocculation and/or coalescence (van der Ven, Gruppen, de Bont & Voragen, 2001). These phenomena accelerate the creaming or oiling-off, which in turn reduce the shelf life of emulsions. Figure 3.7 shows the changes in CI of WPC- and TWPC-stabilized emulsions during storage at room temperature for up to 14 days. Except for emulsions made with 1% (w/w) protein, all the texturized samples formed significantly stable emulsions compared to the controls, as indicated by lower CI values of the former. The stability of TWPC-stabilized emulsions was greatly influenced by protein concentration, increasing the protein level enhanced emulsion stability. In contrast, the non-texturized WPC emulsions were prone to creaming regardless of the protein levels used. The emulsions were completely destabilized after one day of storage, as observed by the plateau in CI values throughout the storage period.

At 2.5% protein concentration, creaming was not observed at day-1 of storage in TWPC-0.3 and TWPC-0.6 stabilized emulsions, while a CI value of 7.9% was obtained in TWPC-S emulsion. During prolonged storage for up to 14 days, CI values rose steadily due to continued breakdown of the emulsions. TWPC-S emulsions were the least stable with CI values of 31.3 and 42.2% at days 7 and 14, respectively. No significant different was observed between TWPC-0.3 and TWPC-0.6, in which the CI values ranged from 15.9 to18.2% and 23.5 to24.8% at day-7 and day-14, respectively. All the TWPC emulsions prepared with 4% protein remained stable throughout the test period. Several authors (Firebaugh & Daubert, 2005; Rosa, Sala, van Vliet & van de Velde, 2006) have previously demonstrated a similar, positive correlation between protein concentration of polymerized WPI and emulsion stability.

The observed behavior of TWPC-based emulsions may be a function of enhanced continuous-phase viscosity of the emulsions that retarded the movement of fat droplets. Increasing protein content also provided more protein to coat the fat droplets, which resulted in increased droplet density, hence minimizing the tendency for phase separation. The lower emulsion stability of TWPC-S can possibly be attributed to the presence of starch that induced depletion and flocculation, leading to fast serum separation and consequently a decrease in emulsions stability (Chanamai & McClements, 2001; Dickinson et al., 1994; Ye, Hemar & Singh, 2004).

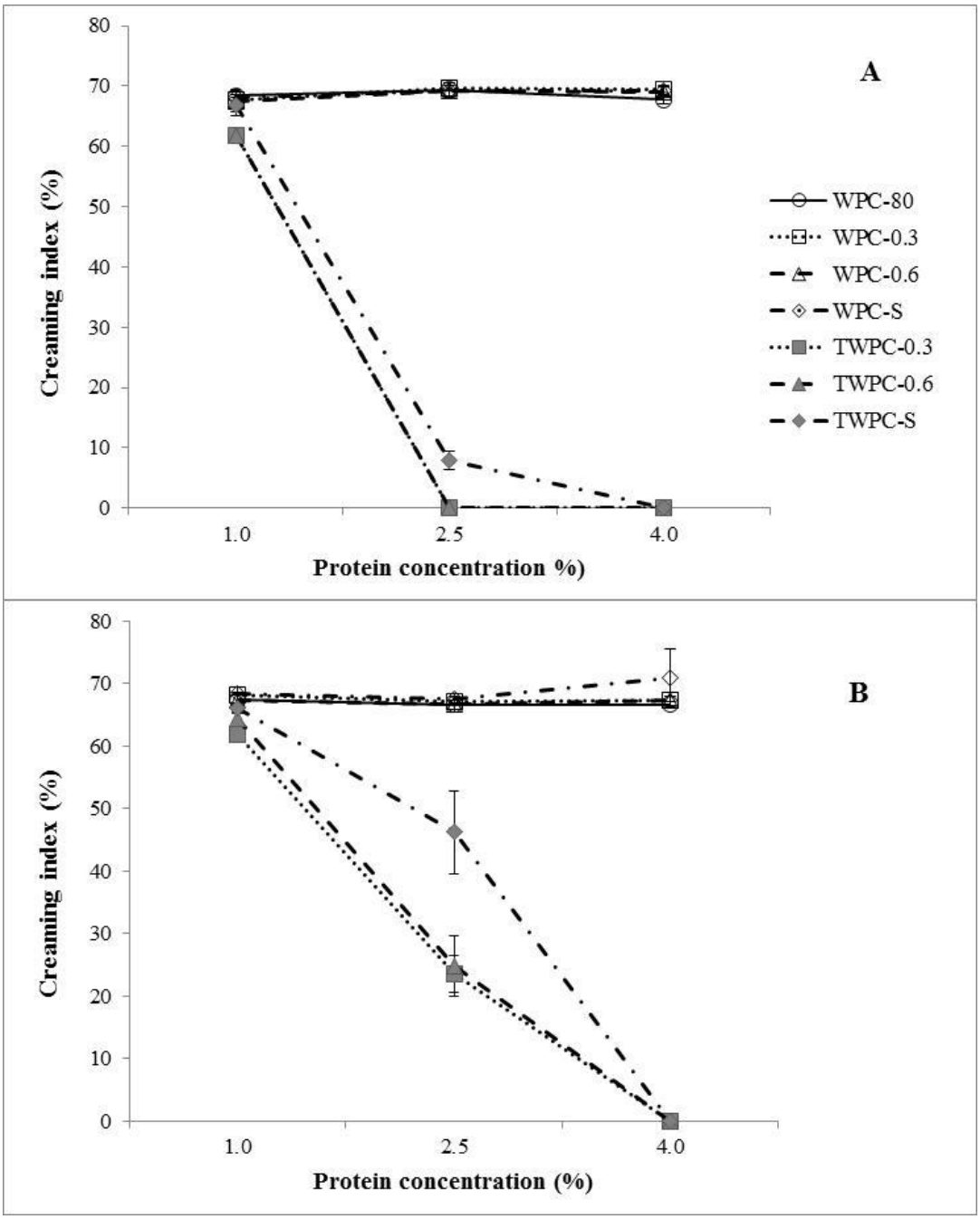

Figure 3.7. Creaming index (%) of emulsions stabilized by WPCs (controls) and TWPCs at various protein concentrations stored for (A) 1 day, and (B) 14 days at room temperature.

3.4.7. Scanning electron microscopy (SEM) of cold-set gels

Figure 3.8 shows the SEM images of cold-set TWPC gels (20%, w/w). The non-texturized WPCs were not analyzed because the samples were in liquid state, which does not support the fixation and drying processes. The SEM micrographs indicated the presence of small, spherical protein aggregates. The gels had coarse microstructures with the presence of pores throughout the gel networks. The gels formed by TWPC-0.3 and TWPC-0.6 were more homogenous than TWPC-S gel that showed the presence of void space and discontinuity. However, the 'continuous' part of the gels presented a more compact and denser network with smaller pore size. The heterogeneous structure of TWPC-S may perhaps be attributed to the presence of starch particles that were embedded in the continuous protein matrix. Similarly, several authors (Olsson, Stading, & Hermansson, 2000; Olsson et al., 2002) have demonstrated that mixed β-lg–high amylopectin starch gel had inhomogenous network consisting of small and large pores compared with pure β-lg gel that formed more homogenous structure although the former had greater gel strength. This was attributed to the incompatibility of protein and starch that separated into two different phases, and the formation of thicker protein strands in the protein-rich phase. Large porosity was related to fewer intermolecular crosslinkings (Van Camp, 1997), while a decrease in the number of pores increased the water holding capacity and elasticity of cold-set WPI gels (Khun, Cavallieri & de Cunha, 2010). Therefore, it may be concluded that as the gel structures gets denser, the system becomes capable of holding more water, resulting in greater viscosity as observed in the case of cold-set TWPC-S gels.

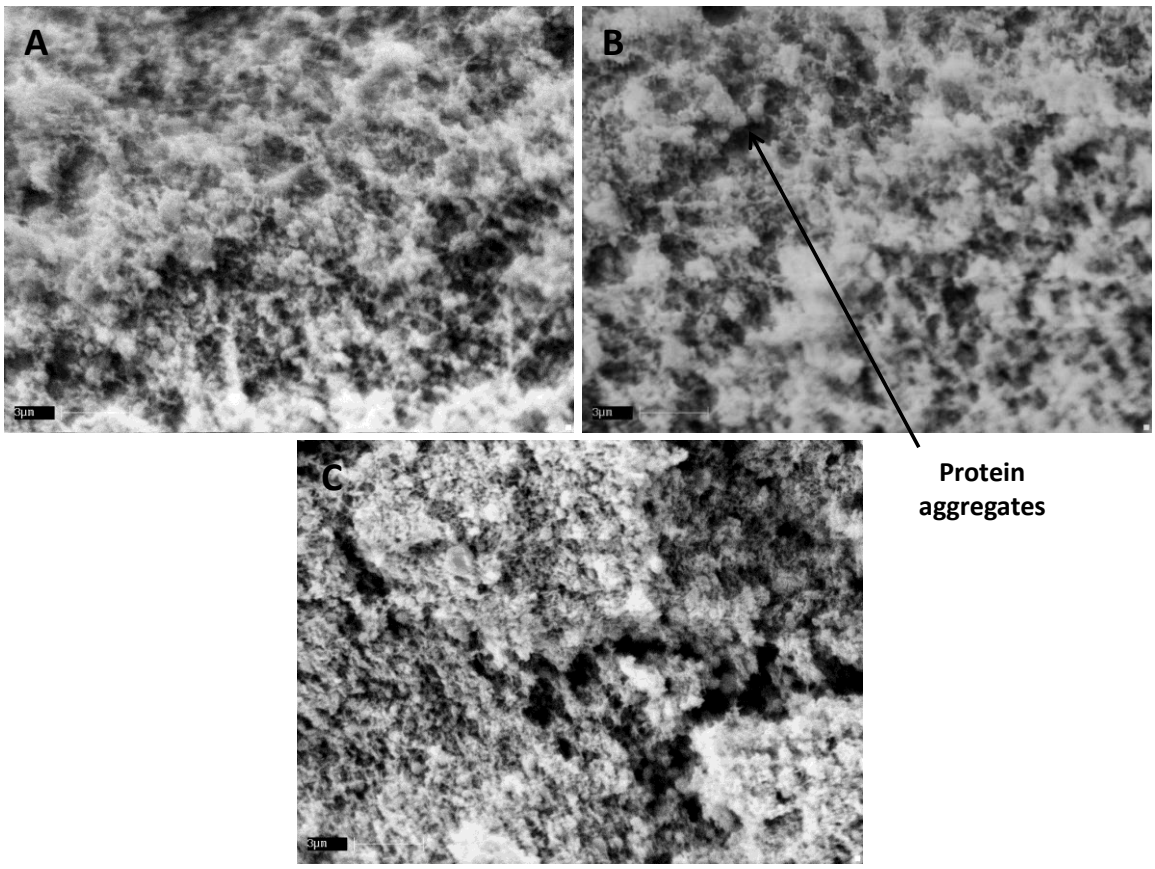

Figure 3.8. SEM micrographs of cold-set TWPC gels (20%, w/w) scanned at 10000x magnification. (A) TWPC-0.3, (B) TWPC-0.6, and (C) TWPC-S. Scale bar corresponds to 3 µm.

3.5. Conclusions

The RSCFX process produced TWPC ingredients with 200- to 300-fold greater viscosity and enhanced emulsion stability than non-texturized WPC. The apparent viscosity of TWPCs increased with increasing protein concentration and formed cold-set gels at 20% (w/w) TWPC. Texturization did not significantly impair the emulsifying activity index of TWPC-stabilized emulsions, but rather greatly improved the emulsion stability attributed to the high aqueous-phase viscosity of the emulsions. The addition of starch resulted in TWPC as an ingredient with greater apparent viscosity but the presence of the added biopolymer impaired the emulsifying properties due to interference with the adsorption of protein at the oil–water interface. Reduced levels of calcium, however, improved viscosity and emulsifying properties. The TWPC cold-set gel network was enhanced during 24 h cold-storage due to strengthening of intermolecular protein interactions. The SEM observations of the cold-set gels revealed a particulate network with a coarse microstructure and the presence of pores throughout the gel network that may well explain their rheological properties. The enhanced functional properties of TWPCs were attributed to the conformational changes in WP molecules as a result of protein denaturation and aggregation that were physically and chemically induced during the RSCFX process.

3.6. References

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CHAPTER 4

THE EFFECTS OF REACTIVE SUPERCRITICAL FLUID EXTRUSION PROCESS ON SURFACE HYDROPHOBICITY OF WHEY PROTEIN CONCENTRATE AND ITS RELATION TO STORAGE AND HEAT STABILITY OF CONCENTRATED EMULSIONS*

4.1. Abstract

Whey protein concentrate was texturized using reactive supercritical fluid extrusion (RSCFX) via a combination of shear, heat, low acidity (pH 3.0) and supercritical carbon dioxide (SC-CO_2). The texturized WPC (TWPC) at 20% (w/w) solution formed a very viscous homogenous paste upon reconstitution with water at room temperature. Texturization increased the aromatic hydrophobicity and decreased the aliphatic hydrophobicity, determined using 8-anilino-1-naphthalene sulfonic acid (ANS) and cis-parinaric acid (CPA) probes, respectively. WP stabilized emulsions containing 40–80% (w/w) oil showed smaller average droplet sizes (4–5 μm) and narrower distributions when TWPC was used as opposed to the non-texturized WPC. The TWPC formed stable emulsions with a gel-like consistency that were stable for 30 days of storage at 4 and 25 °C and showed remarkable heat stability when heated at 70, 80 and 90 °C for 20 min. The improved stability of the TWPC stabilized emulsions was attributed to the interplay of higher protein surface hydrophobicity and greater continuous-phase viscosity. The TWPC with excellent emulsion stabilizing property can be of practical utility in both reduced-fat and high-fat food applications requiring high heat treatments.

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4.2. Introduction

Whey protein (WP) is widely used in food formulations because of its excellent functional and nutritional properties. One of the important functional properties of the WP is the stabilization of emulsion attributed to their ability to adsorb at oil-water interface. The adsorption induces the formation of viscoelastic protein film through a combination of hydrogen bonding, hydrophobic and electrostatic interactions, and sulfhydryl-disulfide interchange reactions (Damodaran & Anand, 1997; Dickinson & Matsumura, 1991; Monahan, German, & Kinsella, 1995). The formation and stability of the emulsions largely depend on molecular interactions of the adsorbed protein and how the interactions are influenced by environmental conditions such as pH, ionic strength and temperature (Dickinson & McClements, 1995). The stability of the emulsions during heat treatment, storage and final consumer use is a highly important characteristic for their utilization in food.

Protein surface hydrophobicity is another important factor associated with emulsion stability and researchers have emphasized its importance while explaining the surface functionality of proteins (Moro, Gatti, & Delorenzi, 2001; Mitideri & Wagner, 2002). The effect of heat treatment on emulsifying activity and stability of various proteins can be explained using the concept of surface hydrophobicity (Voutsinas, Cheung, & Nakai, 1983). Surface hydrophobicity of WPC heated at 85°C for 5 to 60 min was positively correlated with interfacial properties such as emulsifying and foaming (Moro et al., 2001). Combination of acidification and heat treatment improved the foaming and emulsifying properties of proteins due to an increase in surface hydrophobicity induced by deamidation and denaturation (Wagner, Sorgentini, & Anon,

1996). In general, denatured proteins exhibit greater surface hydrophobicity resulting from partial unfolding and thus exposing the hydrophobic regions previously concealed within the native globular structure. The improved emulsifying properties of the denatured proteins was the result of greater film rigidity attributed to the enhanced hydrophobic interactions between adjacent protein molecules that adsorbed at the oil-water interface (Kato & Nakai, 1980; Kinsella & Whitehead, 1989; Mitidieri & Wagner, 2002).

Over the past few years, considerable interest has emerged in converting WP stabilized emulsions into heat-set emulsion gels due to their utility in creating foods with new and improved organoleptic properties (Dickinson & Chen, 1999; Sok Line, Remondetto, & Suburade, 2005). However, heat treatment to a certain temperature/time combination causes aggregation of the fat droplets and destabilizes the emulsions. The state and extent of fat droplet aggregation depend on the inter and/or intra-droplets interactions, which in turn are influenced by factors such as the thickness of protein layer, amount of adsorbed and non-adsorbed proteins, hydrophobicity of both protein molecules and emulsion droplets, and aqueous phase conditions such as pH, ionic strength and temperature (Demetriades, Coupland, & McClements, 1997; Demetriades & McClements, 1998; Dickinson, 2001; Dickinson & Parkinson, 2004; Euston, Finnigan & Hirst, 2000; Jiménez-Flores, Ye, & Singh, 2005; Monohan, McClements, & German, 1996a,b; Sliwinski, Roubos, Zoet, van Boekel, & Wouters, 2003). Furthermore, the high temperature for gel forming conditions prevents the incorporation of thermo-labile compounds into the emulsion gel system (Sok Line et al., 2005). Therefore, cold-gelation of WP stabilized emulsions has been reported as one of the possible alternatives

to overcome the limitations. The cold-gelation of emulsions has been achieved by preheating the WP solution to form soluble aggregates, homogenizing it with oil, followed by acidification (Boutin, Giroux, Paquin, & Britten, 2007; Britten & Giroux, 2001; Rosa, Sala, van Vliet, & van de Velde, 2006) or by addition of salts (Sok Line et al., 2005; Ye & Taylor, 2009) to induce gel formation at room temperature. The process produces stable and homogenous cold-set emulsion gels with predominantly elastic gel behavior and finely dispersed fat droplets (Manoi & Rizvi, 2009; Liu & Tang, 2011).

Our previous work has investigated a new approach for modification of WPC using a novel reactive supercritical fluid extrusion (RSCFX) process, in which the extruder was used as a controlled bioreactor. The process utilizes a combination of low-shear, low-heat (<100°C), low acidity (pH 3.0) and supercritical carbon dioxide (SC-CO_2) to generate texturized WPC (TWPC) that is capable of forming cold-set gel upon reconstitution with water at 20% (w/w) (Manoi & Rizvi, 2008). The TWPC also acted as a good emulsifying agent in that 4% (w/w) TWPC was capable of stabilizing a highly concentrated cold-set emulsion gel containing 80% (w/w) oil. The enhancement of viscosity by incorporation of the texturized protein in the aqueous phase and its excellent emulsifying properties served as the major factors stabilizing the resulting emulsions (Manoi & Rizvi, 2009). In the present study, it was hypothesized that the texturization enhances the surface hydrophobicity of the WP and improves its emulsifying properties, leading to the formation of a stable emulsion. Our objectives were to investigate the effect of RSCFX-based texturization process on surface hydrophobicity of WPC and its relation to storage and heat stability of TWPC stabilized concentrated cold-set emulsion gels.

4.3. Materials and methods

4.3.1. Materials

Commercial WPC-80 (Lactalbumin-49320) was purchased from Leprino Foods Company (Denver, CO, USA). The composition of the WPC was 81.5% protein (dry basis), 5.5% fat, 6.5% lactose and less than 3.0% ash. Pre-gelatinized corn starch (Xpandar® 612) was obtained from Tate & Lyle Ingredients (Decatur, IL, USA). NaCl, $CaCl_2$, Nile red, Fast Green FCF and 8-anilino-1-naphthalene sulfonic acid (ANS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cis-parinaric acid (CPA) and hydrochloric acid (HCl) were purchased from Molecular Probes (Eugene, OR, USA) and JT Baker (Mallinckrodt Baker Inc., NJ, USA), respectively. Commercial soybean oil was purchased from a local store.

4.3.2. Production of TWPC by RSCFX extrusion process

Texturized whey protein concentrate (TWPC) was produced using a RSCFX process reported by Manoi and Rizvi (2009a). Thirty-four kg of commercial WPC-80 was pre-hydrated to 10% moisture (wet basis) by spraying appropriate amount of water to the WPC-80 powder while mixing using a SP130 San Cassiano mixer (Roddi d'Alba, Piemonte, Italy). Pre-gelatinized corn starch was added to the pre-hydrated WPC-80, resulting in a WPC-starch mixture containing 94% WPC-80 and 6% starch. The starch acted as a binder to hold protein matrices due to their ability to form hydrogen bonds in the extruded product (Amaya-Llano, Hernandez, Tostado, & Martinez-Bustos, 2007). Next, salts were added to the mixture to obtain a WPC-starch blend containing 0.6%

(w/w) NaCl and 0.6% (w/w) $CaCl_2$ (based on the WPC-starch basis). Mixing was continued for another hour, followed by overnight storage at room temperature. The preconditioned sample was extruded using a pilot-scale Wenger TX-52 Magnum co-rotating twin screw extruder (Wenger Manufacturing, Sabetha, KS, USA) with a length to diameter ratio (L/D) of 28.5:1 and fitted with two circular inserts of 1.2 mm diameter each. The extrusion process was performed at a screw speed of 180 rpm and feed rate of 35 kg/h. The barrel pressure was maintained at 10–15 MPa for continuous SC-CO_2 injection (at 1.5% dry feed basis) into the protein polymer melt. HCl solution (15%, w/w) was injected into the extruder's mixing zone at 60% moisture content (dry feed basis) to obtain a pH of extrudate of about 3.0. The final product temperature at the die exit was maintained at 90 °C. The extrudate was collected, air dried to 5 to 7% moisture content, and ground to a particle size of equal or less than 1 mm using a mill (Thomas-Wiley Mill model ED-5, Arthur H. Thomas Co., PA, USA). Protein and lipid contents of the TWPC determined by Kjeldahl and Soxhlet methods were 74.0% and 3.2% (dry basis), respectively. The non-texturized WPC-starch blend was used as control throughout the study.

4.3.3. Determination of surface hydrophobicity index

Index of surface hydrophobicity (S_o) was determined by fluorescence spectroscopy method reported by Alizadeh-Pasdar and Li-Chan (2000) using ANS and CPA probes with some modifications. A series of protein solutions (0.005 to 0.025%, w/w) were prepared by diluting a stock protein solution (0.05%, w/w protein) in 0.01 M

phosphate buffer (pH 7.0). ANS solution (8 mM in 0.1 M phosphate buffer, pH 7.0) and CPA solution (3 mM in ethanol) were used to determine aromatic and aliphatic hydrophobicity, respectively. Twenty microliter of the probe solution was added to 4 mL of each serial protein dilution. Solution was vortexed and equilibrated in dark for 15 minutes before relative fluorescence intensity (RFI) measurement was determined using SLM 8000 Spectrofluorimeter (SLM AMINCO Instruments, Inc, Rochester, NY, USA). Fluorescence intensity was recorded at 390 nm and 325 nm for excitation and at 470 nm and 420 nm for emission, for ANS and CPA, respectively. The excitation and emission slit widths were 4 and 4 nm, respectively for ANS and 2 and 4 nm, respectively, for CPA. The index of surface hydrophobicity was determined from the initial slope of the plot of fluorescence intensity vs. protein concentration. Within the protein concentration range used in these experiments, linear relationships were obtained (R^2 = 0.98-0.99).

4.3.4. Emulsion preparation

Continuous phase of emulsion was prepared from a fixed concentration (20%, w/w) of non-texturized WPC or TWPC, by stirring appropriate amount of protein in deionized water for 2 hours at room temperature (25 °C), followed by overnight storage at 4 °C. After two hours of mixing, the TWPC solution formed a homogenous, smooth and highly viscous paste, whereas control sample was a shear thinning liquid. The apparent viscosity (measured by steady shear ramp test at 25 °C) of the TWPC paste at 25 s^{-1} was 3.74 Pa, whereas it was 0.01 Pa.s for non-texturized WPC solution and the pH of each was 3.0 and 6.2, respectively. Emulsions containing 40, 50, 60, 70 and 80%

(w/w) soybean oil were prepared by weighing appropriate amount of protein pastes/solutions and oil in glass containers followed by mixing using a high-speed dispersing unit (IKA Ultra Turrax, T25 basic, IKA Works, Inc, NC, USA) at 11 000 rpm for 3 min at room temperature (25 °C). In the case of emulsions containing ≥ 60% oil, a different method was employed due to inability to mix a highly concentrated emulsion using the high speed mixer attributed to their very viscous nature. A pre-emulsion containing 50% oil was prepared as described above, followed by the addition of remaining oil at 10 mL/min while mixing using a Toastmaster beater (Toastmaster Inc., MO, USA). Sodium azide (0.02%, w/w) was added to prevent microbial growth. The addition of oil up to 80% (w/w) did not change much the final pH of both non-texturized WPC and TWPC emulsions from the initial pH of the protein pastes/solutions used.

4.3.5. Rheological behavior of emulsions

Steady shear ramps of emulsions containing 40 to 80% (w/w) oil were performed using a strain-controlled rheometer (ARES, TA Instruments, New Castle, DE, USA) equipped with a cone and plate geometry (25 mm diameter, 0.051 mm gap and 0.1 radians cone angle). The shear rate was ramped from 1.0 to 100 s^{-1}. All the measurements were performed at 25 °C. The flow curve was fitted to Power Law model;

$$\eta_a = K\dot{\gamma}^{n-1} \quad (1)$$

where η_a is apparent viscosity (Pa.s), K is consistency index ($Pa.s^n$), $\dot{\gamma}$ is shear rate (s^{-1}) and n is flow behavior index.

4.3.6. Storage stability of emulsion

To examine the storage stability of the emulsions as a function of time and temperature, the emulsions were divided into two parts and stored at 4 and 25 °C, respectively. Visual and microstructural observations of the emulsions using confocal laser scanning microscopy (CLSM) were performed for fresh emulsions (day 0) and emulsions stored for 1, 7, 14, 21 and 30 days.

4.3.7. Heat stability of emulsion

Freshly prepared emulsions containing 40, 60 and 80% oil (w/w) were heated in water bath at 70, 80 and 90 °C for 20 min. and immediately placed in ice bath to cool down the samples to room temperature. The microstructures of the non-heated and heat treated emulsions were investigated using CLSM.

4.3.8. Confocal laser scanning microscopy (CLSM)

Microstructures of emulsions were investigated using a confocal laser scanning microscopy (Leica TCS-SP2, Leica Microsystem Inc., Bannockburn, IL, USA). The emulsions were stained with a mixture of Nile Red (0.01%, w/w) prepared in polyethyleneglycol, glycerol and deionized water in a proportion of 50/45/5 and Fast Green FCF (0.001%, w/w) dissolved in deionized water. The Nile Red, a hydrolytic oxazone dye that is specific to triglycerides (Greenspan, Mayer & Fowler, 1985) was used to visualize the dispersed oil phase that appeared as bright green globules. The Fast

Green FCF that is electrostatically attracted to charged groups on proteins (Merril & Washart, 1998) was used to visualize the water/protein phase that appeared as dark or red background. A 40.0x HCX PL/1.25 oil immersion objective lens with additional 8x zoom was used. The green emission range was 500–580 nm and red emission range was 650–730 nm. Confocal illumination was provided by an Argon laser with excitation at 488 nm and a Helium Neon laser (HeNe) with excitation at 633 nm. The size of fat droplets was estimated using MetaMorph Imaging software Series 7.1.4 (Universal Imaging Corp., PA, USA) based on the calculation of the diameters of 200 to 350 fat droplets for each image. A minimum of three images were selected for each emulsion.

4.3.9. Statistical analysis

Statistical analysis was performed using MINITAB (Version 16) (State College, PA). Difference between means was compared using Tukey's test ($p< 0.05$). Triplicate measurements were made for all tests.

4.4. Results and discussion

4.4.1. Surface hydrophobicity

Changes in aromatic and aliphatic hydrophobicity following the RSCFX process are illustrated in Table 4.1. The index of aromatic surface hydrophobicity (S_o) of TWPC measured using ANS was 397 ± 16.19, that was significantly ($p<0.05$) higher by 57.54% compared to non-texturized sample. The aliphatic hydrophobicity of the control WPC as

quantified by CPA showed a 35.61% decrease from 4027 to 2593 following the texturization process. As is frequently observed, thermal denaturation of β-lactoglobulin (β-lg), WPC and WPI solutions heated (above 70 °C) at acidic and neutral pH causes a nearly two-fold increases in aromatic surface hydrophobicity of the proteins (Alizadeh-Pasdar & Li-Chan, 2000; Hayakawa & Nakai, 1985; Moro et al., 2001). Silva, Arêas, Silva and Arêas (2010) reported an increase in ANS protein hydrophobicity following extrusion of soy protein at 130 °C with 35% moisture content as a result of molecular reorganization of protein. An approximately ~31% decrease in aliphatic hydrophobicity has been reported for WPC heated at 80 °C in the presence of 0.03 M $CaCl_2$ (Voutsinas et al., 1983). The findings reflected a significant change in ANS and CPA access to the hydrophobic sites on the protein molecules as a result of the modification process. This behavior was likely a consequence of the changes in the conformation of β-lg that contains a high proportion of hydrophobic amino acid side chains (Laligant, Dumay, Valencia, Cuq, & Cheftel, 1991). The increase in ANS binding was attributed to the exposure of aromatic hydrophobic sites following the modification process (Iametti, Cairoli, De Gregori & Bonomi, 1995; Nir, Feldman, Aserin & Garti, 1994), whereas the loss in CPA binding sites was perhaps attributed to the greater intramolecular interactions among the aliphatic groups (Yang, Powers, Clark, Dunker, & Swanson, 2002). Therefore, it can be suggested that the combined effect of intense shear and heat inside the extruder barrel during the RSCFX process transformed the protein conformations, leading to the exposure of aromatic hydrophobic amino acids, while concomitantly causing the burial of some of the aliphatic hydrophobic groups.

Table 4.1. Index of surface hydrophobicity (S_o) of non-texturized WPC (control) and TWPC.

Samples	ANS	CPA
Non-texturized WPC	252^{b} (4.09)	4027^{a} (60.24)
TWPC	397^{a} (16.19)	2593^{b} (232.52)

* The values that do not bear the same letter in the same column are significantly different ($p<0.05$). Values in parentheses show the standard deviation of triplicate measurements.

4.4.2. Steady shear rheology of emulsions

Increasing the dispersed phase concentrations (40 to 80% oil) increased the apparent viscosities of both the TWPC and non-texturized WPC stabilized emulsions. The viscosities of the TWPC stabilized emulsions were significantly higher than those of the non-texturized samples by 2.2 to 42.2 folds at similar oil levels (data not shown). The main contributing factor to the differences in final emulsion viscosity was the greater apparent viscosity of 20% (w/w) TWPC paste that formed the continuous phase of emulsions, compared to the non-texturized counterpart. All the samples tested exhibited a shear thinning behavior, in which TWPC showed a more pronounced shear thinning (n =0.068 - 0.166) compared to the control samples (n = 0.218 – 0.954). At 40 to 60% oil,

TWPC formed paste-like emulsions and soft-solid gel-like emulsions at 70 and 80% oil levels. In contrast, the non-texturized WPC formed liquid emulsions at 40 to 60% oil and a highly viscous emulsion when the oil concentration was increased to 80%. The increase in emulsion viscosity as oil level was increased implied that the droplets not only fitted into the gel matrix as active fillers, but also acted as anchor points to reinforce the network through interactions between protein molecules at the interface and those in the gel matrix (Rosa et al., 2006; Sok Line et al., 2005; van Vilet, 1988).

These results are supported by Figure 4.1, in which a gradual increase in consistency index of the emulsions was observed as the oil levels were increased from 40 to 60%, followed by a steep increase when the oil levels were increased to ≥70%. It was proposed that at ≥ 70% oil, the systems were closely packed, which restrict the movement of the fat droplets, hence drastically increased the system's consistency. This was in agreement with confocal micrographs of the emulsions (Figure 4.2), which show that the emulsions were constructed by a loose network of spherical droplets that progressed to form a compact matrix as the oil level was increased to ≥ 70%. The findings were in line with the observations by Hemar and Horne (2000) for caseinate-stabilized emulsions in which the spherical, close packing limit were shown to fall around 70% oil. The authors reported hexagonal and pentagonal structures in emulsions with a high dispersed phase volume. Therefore, it can be suggested that the highly viscous nature of TWPC stabilized emulsions can be advantageously exploited in the manufacture of spread-like reduced-fat and gel-like high-fat foods.

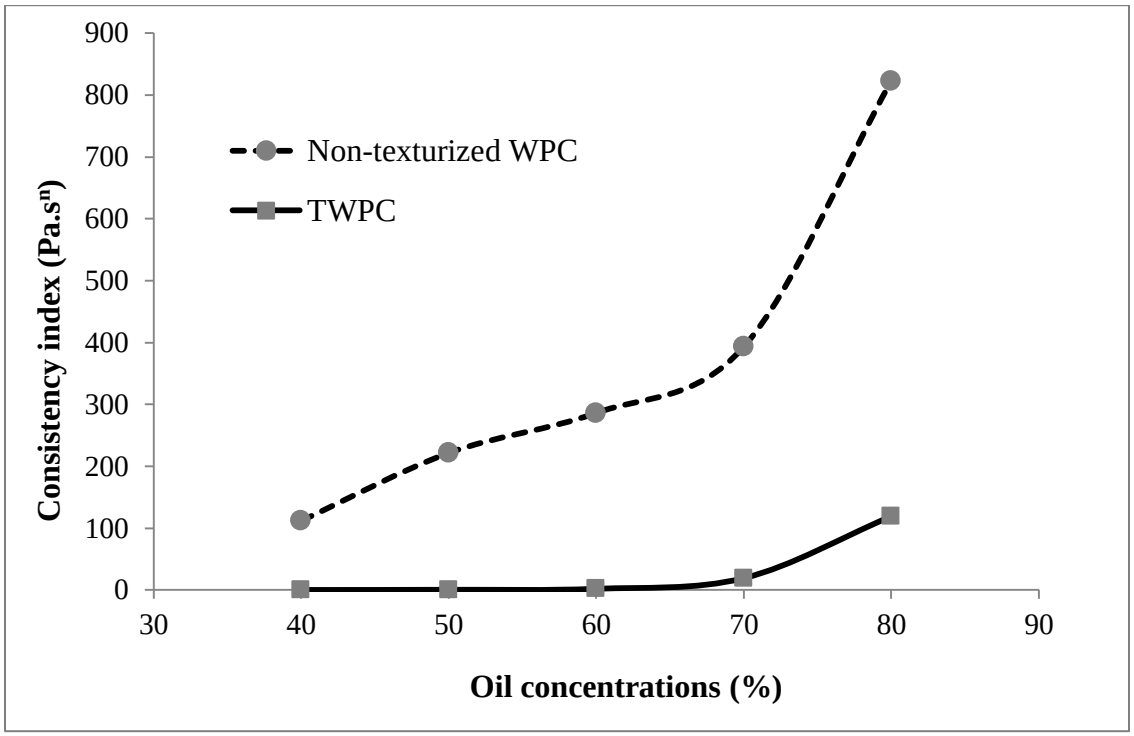

Figure 4.1. Changes in consistency index of non-texturized WPC and TWPC stabilized emulsions at increasing oil levels.

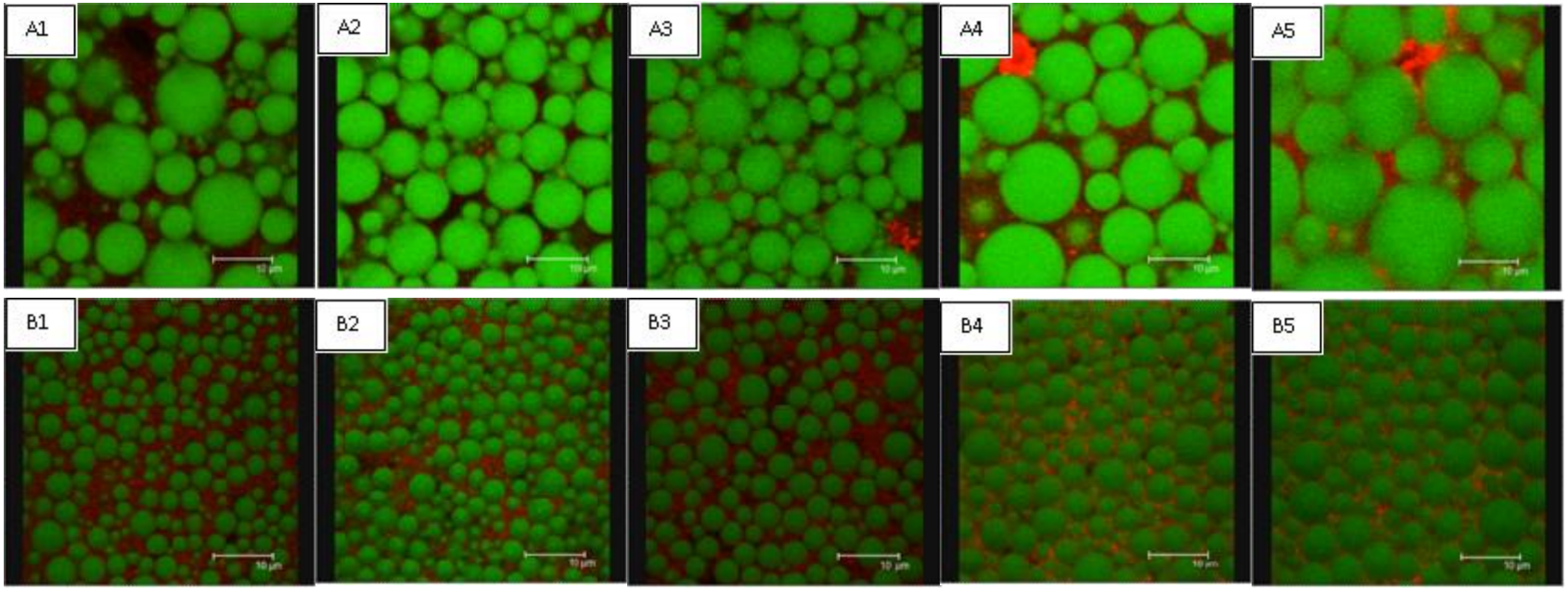

Figure 4.2. CLSM images of (A) non-texturized WPC and (B) TWPC stabilized emulsions containing (1) 40%, (2) 50%, (3) 60%, (4) 70% and (5) 80% oil (w/w) at day-0 (Scale is 10 µm).

4.4.3. Storage stability of emulsions

Figure 4.2 shows that the TWPC stabilized emulsions have significantly smaller average fat droplet compared to their non-texturized counterparts at any oil level tested. The percentage of the fat droplets in the range of 4–5 µm in the TWPC-based emulsions at all oil levels fell between 60.4 to 81.6%, whereas only 12.7 to 29.5% of the droplets of the non-texturized WPC stabilized emulsions fell within this range. An approximately ~50% of the droplets in the former were in the range of 6–9 µm (Table 4.2). The droplets of the TWPC emulsions were homogenously distributed with a narrower distribution, whereas a broader, polydisperse distribution was observed for the control samples (Figure 4.3). The droplet size distribution curves for the latter showed multiple peaks and shoulders. The observed behavior could be due to the increased efficiency of the TWPC to adsorb onto oil-water interface. In general, regardless of protein types, an increase in the fat droplet size and broadening of its distribution were observed with increasing oil levels. This was due to the lower amount of the available proteins to coat the fat droplets. As the oil levels were increased from 40 to 80% (w/w), the WP levels in the emulsions decreased from 12 to 4% (w/w) at 2% interval. Similar findings have been reported by several authors (Desrumaux & Marcand, 2002; Floury, Desrumaux & Lardières, 2000; McClements, 2004).

Table 4.2. Percentage of droplet size[1] of non-texturized WPC and TWPC stabilized emulsions containing different levels of oil at 0 day.

Oil levels (%)	Percentage of droplet size[2] (%)	
	Non-texturized WPC	TWPC
40	29.5 [a]	81.6 [a]
50	12.7 [c]	78.9 [b]
60	27.0 [ab]	65.3 [abc]
70	15.3 [bc]	61.3 [bc]
80	12.0 [c]	60.4 [bc]

[1] Percentage of droplet size in the range of 4–5 µm.

[2] The values that do not bear the same letter in a same column are significantly different ($p<0.05$).

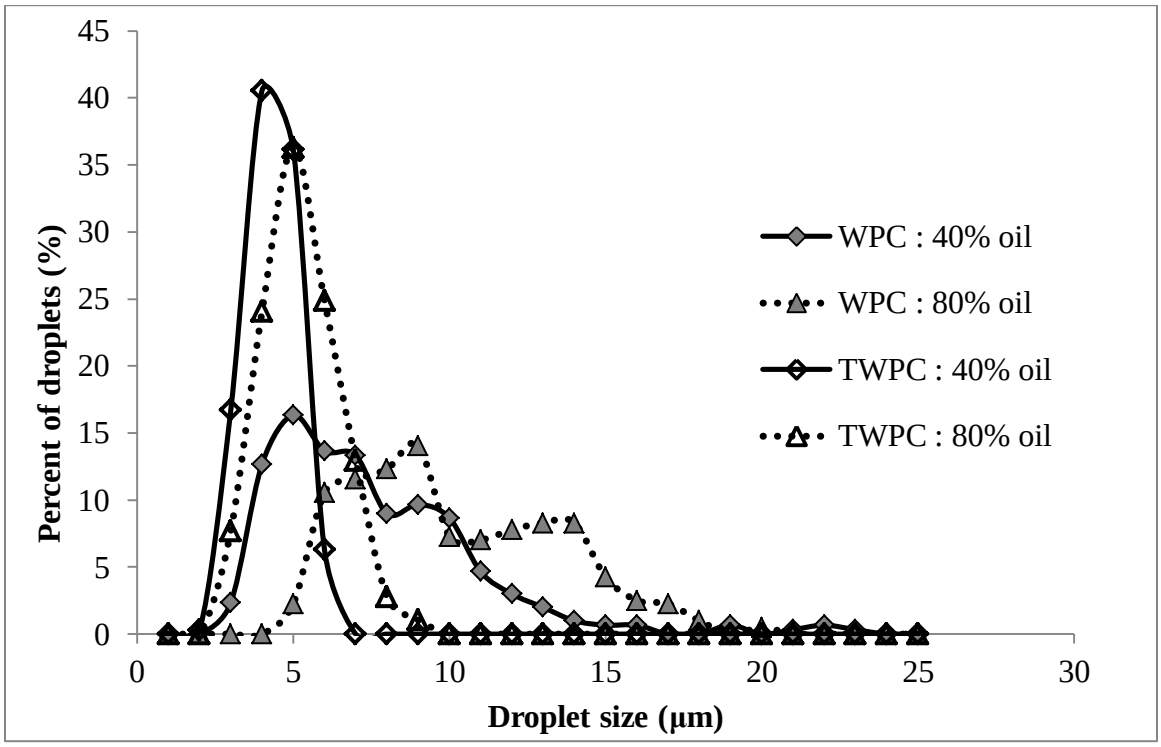

Figure 4.3. Droplet size distributions of non-texturized WPC and TWPC stabilized emulsions containing 40 and 80% oil (w/w) at day-0.

In this study, stable emulsions were defined as those that showed no significant changes in the oil droplets size and/or had no creaming or oiling off following storage or heat treatment. The stability of the emulsions was visually and microscopically observed during 30 days of storage at 4 and 25 °C. Over the storage period, all the non-texturized WPC stabilized emulsions, except for the emulsions containing 80% oil, were unstable against creaming at both 4 and 25 °C. An increase in oil levels decreased the rate and extent of creaming. The creaming was observed at 1, 7, 14, and 21-days of storage at both 4 and 25 °C for emulsions containing 40, 50, 60 and 70% oil, respectively. In

contrast, no creaming was observed in all the TWPC stabilized emulsions that may be predominantly attributed to the highly viscous continuous phase, resulting in the formation of a strong protein gel network. Emulsions that were stable against creaming were microscopically examined for the droplet size distributions. The percentage of droplets in TWPC emulsions in the range of 4–5 μm did not significantly change over the storage period at 4 and 25 °C, and ranged from 78.9–80.9% and 51.6–60.4% in emulsions containing 50 and 80% oil, respectively (Figure 4.4). In contrast, the percentage of droplets (in the range of 4–5 μm) in non-texturized WPC emulsions (80% oil) decreased as much as 20.2 to 77.4% following storage at both temperatures. Even though the percentagees of the droplets (4–5 μm) in control emulsions were not significantly different ($p<0.05$) between 14 and 30-days at both temperatures, however microscopic observations at 30-day of storage indicated a considerable increase in droplet size to 25 μm. The decrease in the percentage of droplets in the reported range indicated a shift to a larger size due to droplet coalescence. The observed differences between TWPC and non-texturized WPC were perhaps due to a greater stabilizing effect of the protein in the former. Similarly, Kiokias and Bot (2005) demonstrated a relatively stable (<30% droplet increase) pre-heated acidified WPC stabilized emulsions during storage at 5 °C over a period of 60 days which was independent of total protein concentration.

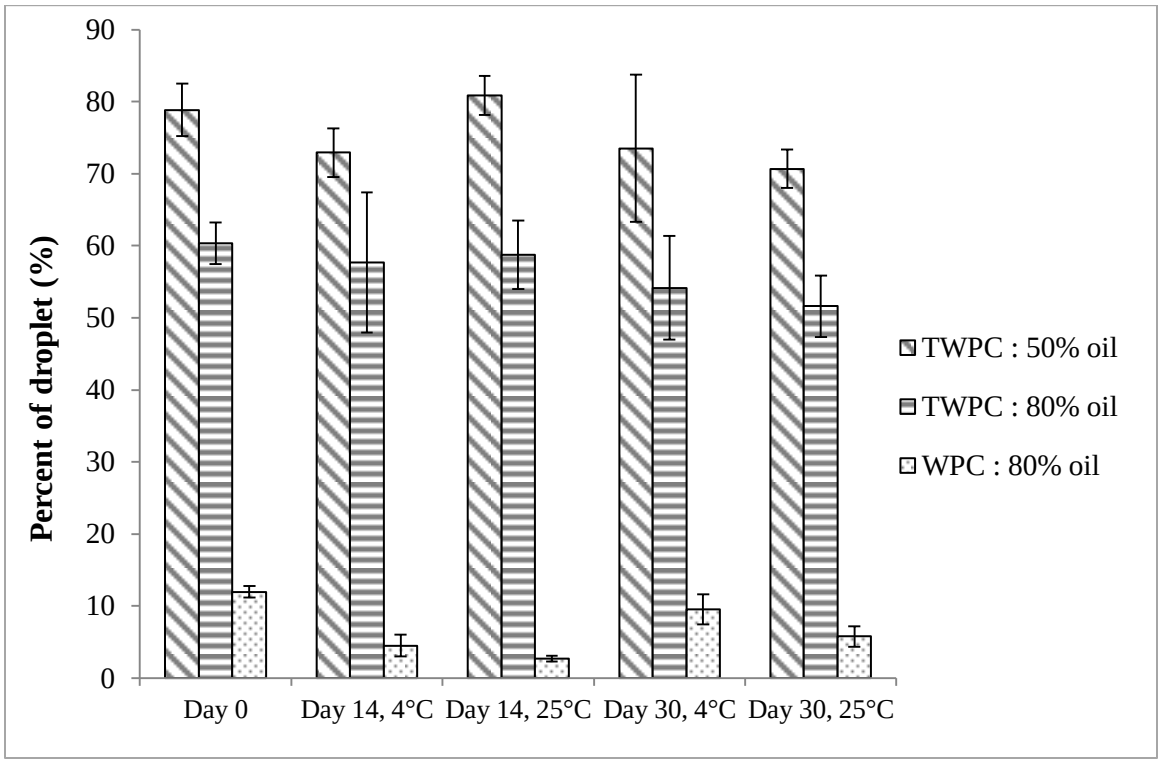

Figure 4.4. Percentage of droplet size of non-texturized WPC and TWPC stabilized emulsions containing 50 and 80% oil at 0, 14 and 30 days of storage at 4 and 25 °C. Percentages of droplet reported are in the range of 4–5 µm. Data not reported for non-texturized WPC emulsions containing 50% oil because the emulsions were phase separated during storage.

Based on these findings, it can be proposed that the enhanced viscosity of continuous phase, higher oil levels and enhanced aromatic hydrophobicity, were the main factors regulating the stability of TWPC stabilized emulsions upon storage. The combined effects of the first two factors predominantly affected the stability of emulsions against creaming. The enhanced viscosity of the continuous phase allowed for the

formation of a weak gel matrix that entrapped the fat droplets within the network, whereas increased oil levels enhanced the interactions between the droplets and protein matrix, retarding the movement of the droplets (McClements, Monohan, & Kinsella, 1993; Patel & Kilara, 1990; Rosa et al., 2006). The greater number of exposed aromatic hydrophobic residues caused an increase in the affinity of the protein molecules towards oil-water interface. This will cause a stronger protein-lipid interaction, which simultaneously shortens the time required to reduce surface tension, hence results in smaller droplets. Smaller droplets have been reported to impart greater stability to the emulsions as they decrease the separation distance between the droplets that leads to an increase in the interactions between protein molecules at the surface (McClements et al., 1993). In addition, the greater surface hydrophobicity may enhance the interaction between adjacent protein molecules that adsorbed at the interface, forming rigid and thick protein membrane, hence providing a greater steric stabilization to the emulsion droplets. Enhanced emulsification of heat denatured soy proteins and WPC has been proposed as a result of the exposure of the buried hydrophobic groups. The higher protein hydrophobicity leads to a greater affinity and stronger adsorption between protein molecules and dispersed phase and thus the formation of thicker protein interfacial layer (Dissanayake & Asiljevic, 2009; Mitidieri & Wagner, 2002; Nir et al., 1994; Surh, Gu, Decker, & McClements, 2005; Surh, Ward, & McClements, 2006). In contrast, the non-texturized WPC that consists of highly structured and globular protein molecules (Graham & Phillips, 1980) with low surface hydrophobicity, possibly formed thinner and less rigid protein membrane that was prone to extensive coalescence during storage.

4.4.4. Heat stability of emulsions

Visual observations of the non-texturized WPC stabilized emulsions showed an increase in consistency upon heating at 70, 80 and 90 °C for 20 min, forming soft solid-like pastes/gels. This was expected because heating the WP above denaturation temperature (> 65 °C) caused the adsorbed proteins to aggregate and cross-link not only with each other but also with the free proteins in the bulk phase through a combination of non-covalent interactions (Hoffmann, Roefs, Verhul, van Mil & de Kruif, 1996). As a result, a protein gel network is formed leading to incorporation of fat droplets into the network, subsequently reinforcing the gel strength (Chen, Dickinson, Langton, & Hermansson, 2000; Jost, Baechler, & Masson, 1986; McClements et al., 1993; Sliwinski et al., 2003). However, no significant changes were observed in the consistency of the heated TWPC stabilized emulsions which may be attributed to the extensively denatured proteins that rendered them high heat stability.

Figure 4.5 shows the changes in droplet size distributions of the samples following heating. The droplet size distributions of the emulsions were observed to be a function of protein type, oil concentration and heating temperature. The non-texturized samples containing 40 and 60% oil showed a substantial stability upon heating at 70 °C. However, a significant increase in droplet size was observed when the emulsions were heated at 80 °C, as indicated by a broadening of the droplet size distribution. Both of the non-texturized WPC and TWPC emulsions containing 40 and 60% oil exhibited a 6.7 and 28.7% decrease in the droplet size of 4 μm, respectively, indicative a greater destabilization occurred in the former.

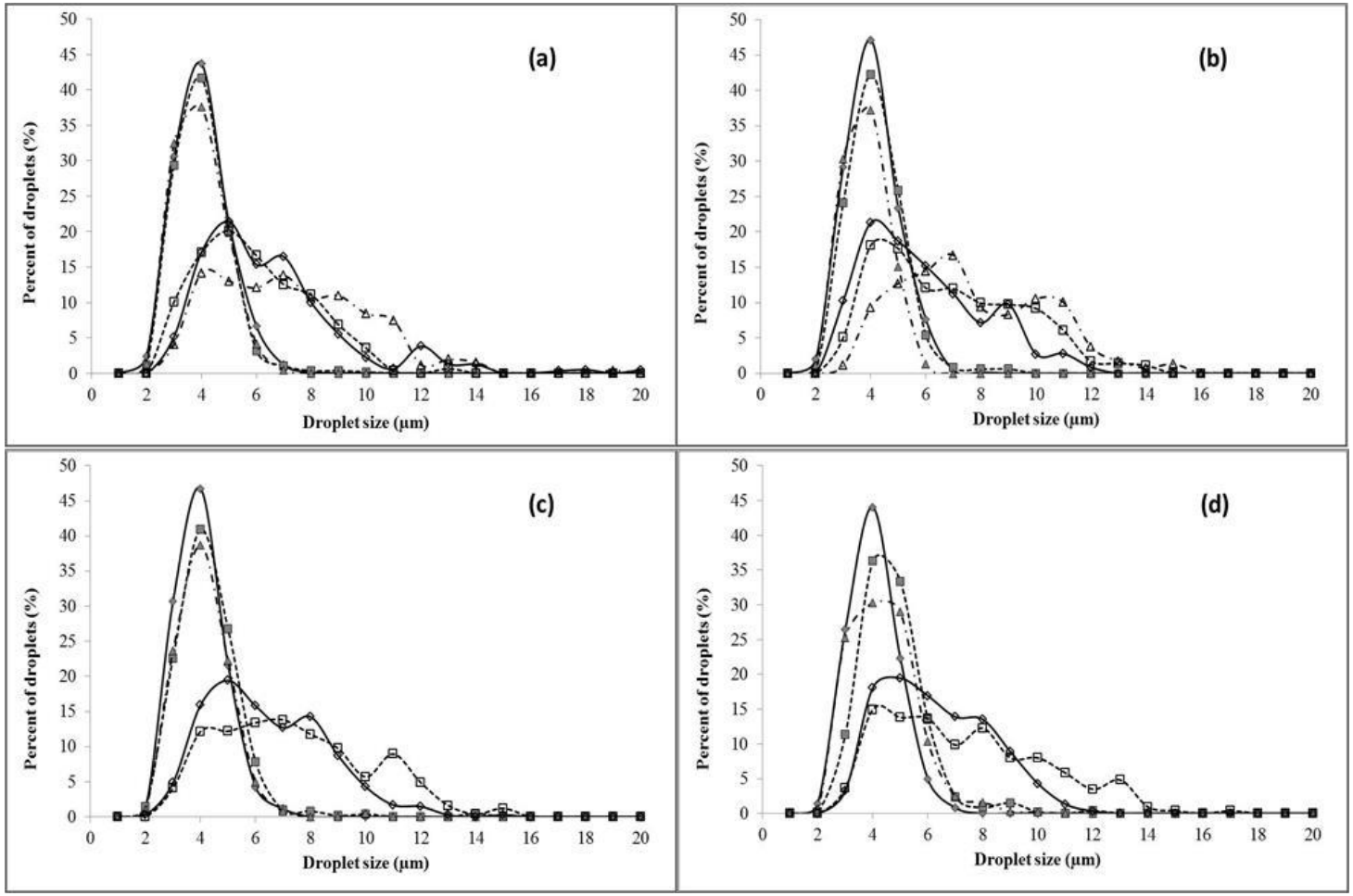

Figure 4.5. Changes in droplet size distributions of non-texturized WPC (open symbols) and TWPC (filled symbols) stabilized emulsions containing 40% (◊), 60% (□) and 80% (Δ) oil (w/w) upon heat treatment. (a) non-heated, and heated at (b) 70 °C, (c) 80 °C, and (d) 90 °C for 20 min.

The droplet size of 4 µm was reported because the highest percentage of droplets in TWPC emulsions fell within this size. Heating both emulsions at 90 °C showed a slight improvement in the emulsion stability compared when heated at 80 °C. Nevertheless, the non-texturized samples containing 80% oil were unstable upon heating at any temperature. A broadening of droplet size distribution was observed at 70 °C, whereas, the emulsions were completely destabilized upon heating at 80 and 90 °C as indicated by observable oiling off. As the oil level was increased, the ability of the emulsion to retain its stability perhaps decreased due to lower surface protein load, causing a reduced rigidity of the protein membrane against heat treatment. In contrast, the emulsions made with the texturized sample showed a substantial stability following heat treatment. In general, the droplet size distributions were not significantly changed by heating at 70 and 80°C. However, upon heating at 90°C, a slight broadening of the size distribution was observed in the TWPC emulsions containing 60 and 80% oil, as indicated by a decrease in percentage of droplet size of 4 µm by 13.0 and 19.6%, respectively, compared to the non-heated emulsions. Figure 4.6 illustrates the CLSM micrographs of the emulsions following heat treatment. Extensive droplet coalescence and free oil, indicated by irregular shaped emulsion droplets were observed in the non-texturized WPC emulsions, whereas the fat droplets in TWPC emulsions did not show any obvious changes due to heating. The observed changes in non-texturized WPC emulsions containing 40 and 60% oil and heated at 70 and 80 °C was consistent with the effects of heating temperature on the stability of emulsions demonstrated by Demetriades et al. (1997) and Monohan et al. (1996a). The authors suggested that the stability of heated emulsions correlated with the hydrophobicity of the adsorbed layer and inter- and

intra-droplet protein-protein interactions. High droplet hydrophobicity at 70 °C was a result of partial protein unfolding that favors inter-droplet interactions, whereas, a decrease in droplet surface hydrophobicity at 80 °C enhances intra-droplet interactions. However, the destabilization of emulsion that occurred at 90 °C rules the possibility of lower stabilizing properties of native WPC in concentrated emulsion system.

It has been reported that in emulsions with high oil content (>40% oil), almost all proteins in the system were adsorbed at the interface, leaving very little unadsorbed protein (Chen & Dickinson, 1998). Thus, in the present work, it is assumed that most of the TWPC adsorbed to the surface of the emulsion droplets, with only a very little free protein existing in the continuous phase. Perhaps all the exposed hydrophobic groups of the proteins interacted with the lipid phase, hence reduced surface hydrophobicity of the droplets. This condition would decrease the probability of inter-droplet interactions, hence preventing the droplet from being close to each other and extensively aggregated. In addition, it is suggested that the thicker and rigid protein membrane played a role in enhancing emulsion stability during heat treatment. Emulsions stabilized by WPC with its higher molecular weight have been reported to experience greater stability to heat treatment. This was due to the relatively higher repulsive steric interactions resulting from the increased thickness of protein membrane (Surh et al., 2005, 2006). Therefore, the TWPC can be a practical approach for application in food products that requires high heat treatment without destabilization of the emulsions.

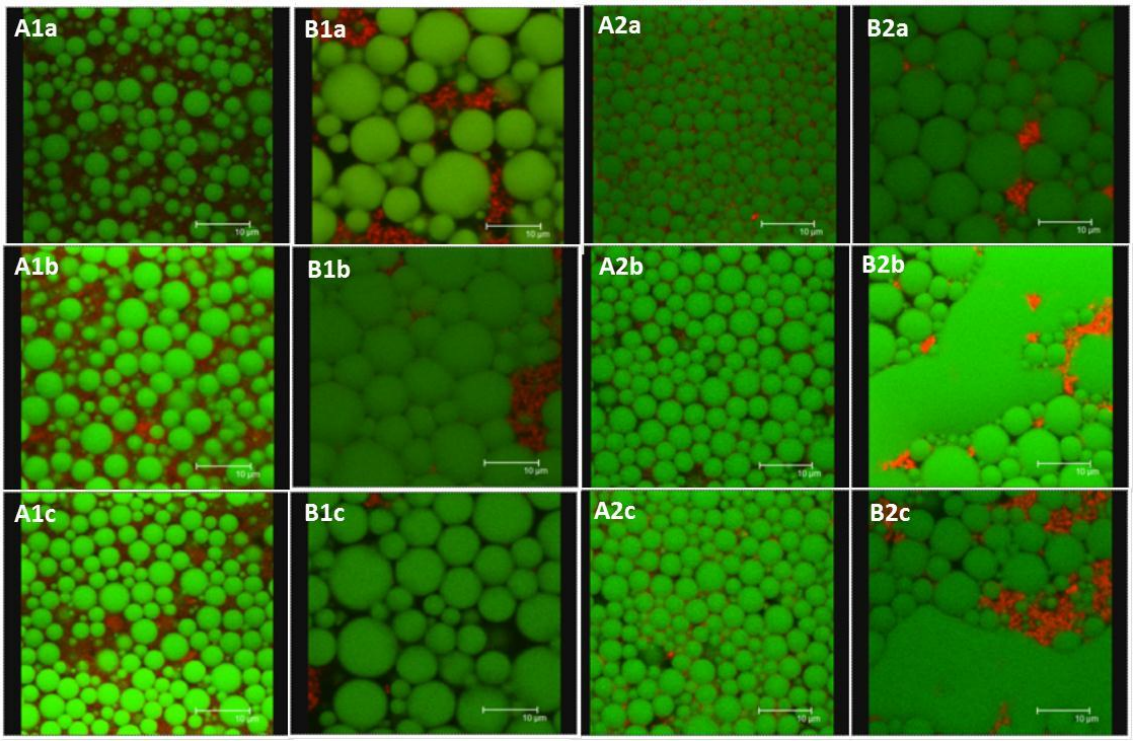

Figure 4.6. Microstructure of heat treated (A) TWPC and (B) non-texturized WPC stabilized emulsions containing (1) 40% and (2) 80% (w/w) oil. The emulsions were heated at (a) 70 °C, (b) 80 °C and 90 °C for 20 min.

4.5. Conclusions

Texturization of WPC via RSCFX process produces a new WPC ingredient that is able to form cold-set protein gels and emulsion gels at room temperatures. The cold-gel setting characteristics are partly due to the increased aromatic hydrophobicity of the texturized protein. In addition, the viscous nature of TWPC forming the continuous phase yielded concentrated emulsions with 40 to 80% oil that were stable during prolonged storage for 30 days at both 4 and 25 °C. The emulsions also showed a considerable stability upon heating to higher temperatures. The stability of the emulsions was a result of interplay between higher protein surface hydrophobicity and enhanced continuous phase viscosity. The TWPC may have formed thicker and stronger protein interfacial layer though the enhanced protein-protein interactions. These new generation of TWPCs will be suitable gelling and emulsifying ingredients for use in both high heat-treated, low-fat and high-fat foods.

4.6. Acknowledgement

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CHAPTER 5

FUNCTIONAL PROPERTIES OF WHEY PROTEIN CONCENTRATE TEXTURIZED AT ACIDIC pH: EFFECTS OF EXTRUSION TEMPERATURE

5.1. Abstract

Reactive supercritical fluid extrusion (RSCFX) was used to generate texturized whey protein concentrate (TWPC) and the impacts of process temperature on product's physicochemical properties were evaluated. TWPC extruded at 50 (TWPC-50) and 70 °C (TWPC-70) formed soft-textured aggregates with high solubility and lower degree of denaturation than that extruded at 90 °C (TWPC-90). Total free sulfhydryl contents and solubility studies in selected buffers indicated that TWPC is stabilized predominantly by non-covalent interactions. Proteins texturized at 90 °C showed an increased affinity for 1-anilino-naphthalene-8-sulfonate (ANS), indicating changes in protein structure. Water dispersion of TWPC at room temperature showed thickening function with shear thinning behavior. Secondary gelation occurred in TWPC-50 and TWPC-70 by heating the cold-set gels to 95 °C. TWPC-90 produced cold-set gels with good thermal stability. Compared to control, TWPC formed stable oil-in-water emulsions. Factors such as the degree of protein denaturation, exposure of hydrophobic groups, and cross-linking influenced the intermolecular associations during cold-set gelation, second-stage heat-induced gelation and emulsion functionalities of the final product. TWPC generated by low and high temperature extrusions can thus be utilized for different products requiring targeted physicochemical functionalities.

5.2. Introduction

The potential of whey protein (WP) as functional ingredient such as gelling, emulsifying, and foaming agents in food is widely acknowledged. The properties of WP vary depending on their intrinsic factors such as extent of protein denaturation, size and shape of protein aggregates, which are themselves affected by their environmental conditions such as pH, ionic strength, and temperature. Heat treatments are commonly used to alter the functional properties of whey proteins and increase their applicability for product applications (deWit 1989; Morr & Ha, 1993). Heat denatures the protein molecules and alters their structures by exposing the hydrophobic and thiol groups buried in the interior of the globular proteins. The extent of exposure of the reactive groups determines the functionalities of the proteins. At a moderate temperature (< 70 °C), the structural unfolding and conformational changes of β-lactoglobulin (β-lg) and α-lactalbumin (α-la) are largely reversible on cooling. At a higher temperature (> 90 °C), the proteins are extensively denatured and irreversibly cross-linked to form large aggregates that vary depending on factors such as pH and ionic strength (de la Fuente, Singh, & Hema, 2002; deWit & Klarenbeek, 1984; McClements & Keogh, 1995). Various mechanisms have been proposed to account for the formation of heat-induced protein aggregates. Non-covalent interactions, mainly hydrophobic, contribute to the formation of protein aggregates in acidic environment, at pH close to the isoelectric point and/or in the presence of high salt concentration, while disulfide bonds predominantly contribute to aggregate formation in neutral or alkaline environments (Galani & Apenten, 1999; Shimada & Cheftel, 1988; Verheul, Roefs, & de Kruif, 1998).

Aggregation of proteins is generally accompanied by a reduction in their solubility. Nevertheless, high solubility is a prerequisite for good emulsifying, foaming, and gelation properties (deWit & Klarenbeek, 1984; Nakai, 1983). A high temperature treatment often leads to irreversible changes in protein structure, which in turns reduces solubility. The solubility of WP is interdependent of pH and temperature: WP loses solubility when heated to < 70 °C at pH 8.0 as compared to heating at pH 4.6 or 6.0. At high temperatures (> 95 °C), solubility is independent of pH (deWit & Klarenbeek, 1984; Li-Chan, 1983). Depending on extrinsic factors, the thermal denaturation of WP results in a heterogeneous mix of native soluble, polymerized soluble, and insoluble protein aggregates (Sanchez, Pouliot, Gauthier, & Paquin, 1997).

For a protein to be an effective emulsifier, it has to form aggregates of an optimum size with balanced surface hydrophobicity and good solubility (Nakai, 1983; Wagner, Sorgentini, & Anon, 1995). Partial denaturation of WP produces soluble, aggregated proteins with increased surface hydrophobicity, which favors protein adsorption at oil–water interface. Extensive irreversible protein aggregation decreases the solubility, resulting in a loss of emulsifying properties (Dickinson & Hong, 1994; Mutilangi, Panyam, & Kilara, 1994). However, whey protein isolate (WPI) or whey protein concentrate (WPC) that contains high amount of denatured protein produces stable emulsions with high viscosity (Britten, Giroux, Jean, & Rodrigue, 1994; Dissanayake, Liyanaarachchi, & Vasiljevic, 2012). The authors described the complementary roles of native and denatured protein in forming the interfacial membranes around oil droplets. In the case of gel formation, increasing gelation time and holding temperature reportedly strengthen the structure of WP gels, due to the increased

hydrophobic interactions and disulfide bondings (McClements & Keogh, 1995; Resch, Daubert, & Foegeding, 2005). Insoluble protein aggregates contribute to gel strength by increasing its water-imbibing capacity (Puyol, Cotter, & Mulvihill, 1999; Sorgentini, Wagner, Arrese, & Anon, 1991). Thus, improving protein functionality requires a compromise between counteracting physicochemical properties of the protein and their careful modulation (Vardhanabhuti, Foegeding, McGuffey, Daubert, & Swaisgood, 2001).

For certain applications, high-heat treatment is not a suitable option for modifying protein to achieve required structural modifications and functionalities. Heat-set gels formed under acidic conditions are brittle and weak, due to weak linkages attributed to the reduction in degree of disulfide cross-linking and pH-associated effects on denaturation reactions (Hudson, Daubert, & Foegeding, 2000; Ramos *et al*., 2012; Shimada & Cheftel, 1989). Therefore, protein ingredients that form gels at room temperature—cold-set gels—are highly desirable. WP has been modified by heating protein solution at 70–90 °C for 5–120 min under low ionic strength and neutral pH conditions to produce soluble protein aggregates. Cold-set gel is formed either by the addition of salt or reduction in pH (Alting, Hamer, de Kruif, & Visschers, 2000; Barbut & Foegeding, 1993). In another studies, cold-gelation of WPI and WPC was formed by reconstitution of derivitized WP (dWP) in deionized water without the salt addition or pH reduction. The dWP powder was obtained from a sequence of process involving thermal gelation (80 °C for 1–3 h) of acidified protein solution (8–12%, w/w), followed by freezing the gel, spray drying or freeze drying and particle size reduction of dried dWP powder (Hudson & Daubert, 2002; Resch, Daubert, & Foegeding, 2004). These gels

have improved water-holding capacity and strength than those produced by conventional heat-induced method (Alting et al., 2000; Barbut & Foegeding, 1993; Hongsprabhas & Barbut, 1996; McClements & Keogh, 1995). However, most of the techniques require high heating temperature with long or multiple steps that may not be practical for commercial production.

Recently, a procedure for producing texturized WPC (TWPC) with enhanced cold-set gelling properties was developed by using a reactive supercritical carbon dioxide extrusion (RSCFX). TWPC processed by RSCFX in the presence of NaCl and $CaCl_2$ under acidic conditions (pH 3.0) showed high water-absorbing capacity and viscosity when reconstituted in water. At 20% (w/w) water dispersion, the proteins formed cold-set gels without the need of salt or acidification (Manoi & Rizvi, 2008). However, during the RSCFX process, proteins are heated to 90 °C, which produces larger protein aggregates and a lower solubility than non-texturized WPC. Because heat treatment has negative impacts on protein solubility, developing a low temperature process that modifies WPC for improved water holding capacity without solubility loss is desirable. Therefore, the objective of this study was to determine the effects of extrusion temperatures on physicochemical functionalities of TWPC.

5.3. Materials and Methods

5.3.1. Materials

Commercial WPC-80 (Lactalbumin-49320) was purchased from Leprino Foods Company (Denver, CO, USA). The composition of the WPC was 81.5% protein (dry basis), 5.5% fat, 6.5% lactose, and less than 3.0% ash. Flourescence probes, 8-anilino-1-naphthalene sulfonic acid (ANS) and cis-parinaric acid (CPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Molecular Probes (Eugene, OR, USA), respectively. Ellman's Reagent (5,5'-dithio-bis-(2-nitrobenzoic acid) was purchased from Thermo Scientific Inc. (Rockford, IL, USA).

5.3.2. Texturization of whey protein by RSCFX

The protein blend was prepared by pre-hydrating WPC-80 to 10% (w/w) moisture. 0.6% NaCl and 0.3% $CaCl_2$ (on weight basis) were added to the pre-hydrated WPC. The premixed protein blend was then preconditioned overnight at room temperature and extruded using a co-rotating twin-screw extruder (Wenger TX-52 Magnum, Sabetha KS, USA) coupled with supercritical carbon dioxide (SC-CO_2) injection system. The extruder, with a L/D ratio of 28.5:1, is configured for the RSCFX process and SC-CO_2 is injected into the barrel through four valves located at L/D ratio of 24. The extrudate was forced through two die inserts with 1.2 mm diameter circular openings. The protein blends were extruded at three different die-exit temperatures: 50, 70, and 90 °C and labeled as TWPC-50, TWPC-70, and TWPC-90, respectively. The barrel zone temperatures were maintained as shown in Figure 5.1; the temperatures of the

second and third barrel zones were adjusted to get products at 50, 70, and 90 °C die temperatures. The extruder was operated at 130 rpm at a feed rate of 35 kg/h. SC-CO_2 (1.5%, w/w of dry feed) was continuously injected at a pressure of 10–15 MPa into the protein melt. HCl solution of 15% (w/w) was injected into the mixing zone to obtain a pH of extrudate of about 3.0 and the extrusion was carried out at 60% moisture. The extrudates were dried at 40 °C for approximately 16 h to achieve 5–6% moisture content. The dried products were finely ground in a mill with a 1.0-mm sieve (Thomas-Wiley Mill model ED-5, Arthur H. Thomas Co., PA, USA), and stored in air-tight containers at room temperature.

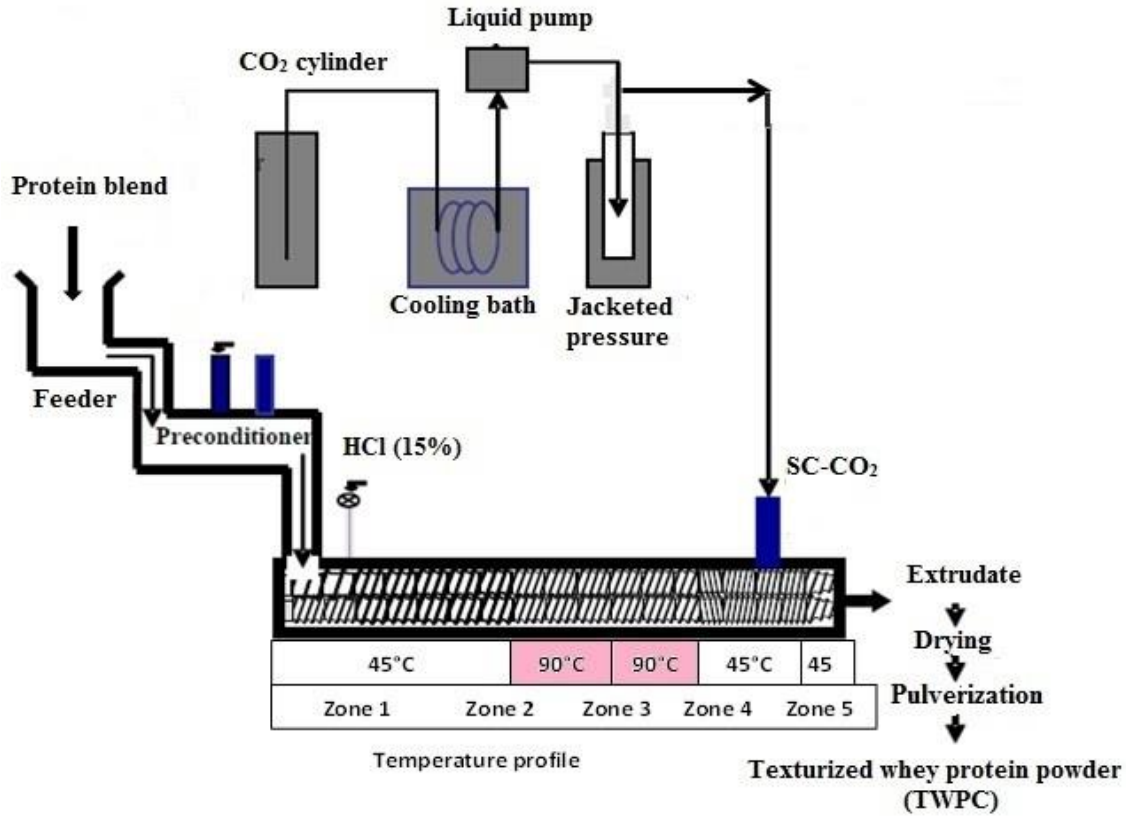

Figure 5.1. Schematic diagram of reactive supercritical fluid extrusion (RSCFX) process. The second and third barrel zones temperatures were adjusted accordingly to get the die-temperatures of 50, 70, and 90 °C.

5.3.3. Particle size distribution

The effective diameter and distribution of protein particles in water dispersion of non-texturized WPC (control), TWPC-50, TWPC-70 and TWPC-90 (1.0%, w/w) were measured with a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corp., Holtsviller, NY, USA). Samples were diluted to 0.10 to 0.05% (w/w) in ultra-pure water, immediately before the particle size measurements. Sample dilution was adjusted in order to achieve signal intensity of 400-800 kilocounts per second (kcps). All measurements were performed at 20 °C. Data was collected and analyzed using BIC software (Brookhaven Instruments Corp.). The intensity-weighed effective diameter (average diameter) and polydispersity index were determined for each sample.

5.3.4. Determination of free sulfhydryl (-SH) groups

The free sulfhydryl groups in protein solutions (1.0%, w/w) was determined using Ellman's reagent, DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid) according to the methods of Sava, van der Plancken, Claeys, & Hendrickx (2005) and Shimada and Cheftel (1989). Protein solutions were diluted to 0.1% (w/w) in buffer containing 0.086 M Tris, 0.09 M glycine, 4 mM Na_2EDTA, 0.5% SDS and 8.0 M urea (pH 8.0). Samples were centrifuged at 20,000 g for 15 min at 20 °C. Thirty microliters of DTNB solution (40 mg DTNB/10 ml buffer) was added to 3 ml supernatant, followed by 15 min incubation at room temperature. The absorbance was recorded at 412 nm using a spectrophotometer (Spectronic 1200, Bausch and Lomb, Rochester, NY, USA). A molar extinction

coefficient of 1.36 x 10^4 $M^{-1}cm^{-1}$ was used to calculate the number of free SH groups, expressed in μmol per gram of protein.

5.3.5. Determination of the index of surface hydrophobicity

Index of surface hydrophobicity (S_o) was determined by fluorescence spectroscopy method reported by Alizadeh-Pasdar and Li-Chan (2000) using 1-anilinonaphthalene-8-sulphonate (ANS) and cis-parinaric acid (CPA) probes, with minor modifications. A series of protein solutions (0.005 to 0.025%, w/w) were prepared by diluting a stock protein solution (0.05%, w/w protein) in 0.01 M phosphate buffer (pH 7.0). The ANS (8 mM in 0.1 M phosphate buffer, pH 7.0) and CPA (3 mM in ethanol) probes were used to determine aromatic and aliphatic hydrophobicity, respectively. Into 4 ml of each protein solution, 20 μl of probe solution was added, and the solution was then incubated in the dark for 15 min. The relative fluorescence intensity (RFI) of the solutions were measured at 390 nm of excitation and 470 nm of emission for the ANS probe and at 325 nm of excitation and 420 nm of emission for the CPA probe, using a SLM 8000 Spectrofluorimeter (SLM AMINCO Instruments, Rochester, NY, USA). The excitation and emission slit widths were 4 and 4 nm, respectively for ANS and 2 and 4 nm, respectively, for CPA. The surface hydrophobicity index was determined from the initial slope of fluorescence intensity vs. protein concentration.

5.3.6. Protein solubility

Protein solubility was determined according to the method of Shimada and Cheftel (1989), with minor modifications. Protein solution (1.0%, w/w) was prepared by dispersing samples in four solutions: (a) deionized water, (b) standard buffer (BF) containing 0.086 M Tris, 0.09 M glycine, and 4 mM Na_2EDTA, pH 8.0 (c) standard buffer containing 8 M urea and 0.5% (17.3 mM) sodium dodecyl sulfate (SDS), pH 8.0 (BFU), and (d) standard buffer containing 8 M urea, 0.5% SDS, and 10 mM dithiothreitol (DTT), pH 8.0 (BFD). Protein dispersions were centrifuged at 20,000 g for 15 min at 20 °C. Protein contents in supernatant (soluble protein) and dispersion (total protein) were determined by bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). The supernatant and protein dispersion were diluted to 0.1% (w/w) before the measurements. Protein solubility was expressed as the percentage of protein in the supernatant to total protein in the initial dispersion.

5.3.7. Steady shear properties

A series of non-texturized WPC and TWPCs solutions/pastes (20–26%, w/w) were prepared by mixing appropriate amounts of powder in deionized water for 2 h followed by overnight storage at 4 °C. A 20% (w/w) solution was used as a starting concentration because TWPC-90 forms a very viscous homogenous paste at this concentration, resembling a weak cold-set gel. Protein solutions made from a range of powder concentrations were tested to determine the minimum concentration of TWPC-50 and TWPC-70 required to achieve viscosity comparable to the 20% (w/w) TWPC-90.

Steady shear rheological measurements were performed with a strain-controlled rheometer (ARES, TA Instruments, New Castle, DE, USA) equipped with parallel plate geometry (50 mm diameter, 1.0 mm gap). The shear rate was ramped from 1.0 to 100 s^{-1} and all measurements were performed at 25 °C. Shear stress and apparent viscosity (η_a) was recorded with TA Orchestrator software. The flow curve was fitted to the Power Law model:

$$\tau = K\dot{\gamma}^n \qquad (1)$$

where τ is shear stress (Pa), K is consistency index ($Pa.s^n$), $\dot{\gamma}$ is shear rate (s^{-1}) and n is flow behavior index. Apparent viscosity was reported at 25 s^{-1}.

5.3.8. Thermal stability of proteins

The thermal stability of non-texturized WPC and TWPCs was determined by strain-controlled rheometer (ARES, TA Instruments, New Castle, DE, USA). A 24% (w/w) protein dispersion/paste was tested using a parallel plate probe (25 mm diameter) at a thickness of 2 mm. A dynamic temperature sweep test was performed by ramping the temperature from 25 to 95 °C at 2 °C/min at a constant frequency of 1 rad/s with 0.1% strain. The storage modulus (G'), loss modulus (G"), and loss tangent (tan δ) were recorded with TA Orchestrator software.

5.3.9. Emulsifying properties

The emulsifying activity index (EAI) and emulsifying stability index (ESI) of the samples were determined by turbidimetric method of Pearce and Kinsella (1978). Emulsions were prepared by mixing 10 mL soybean oil with 40 mL protein dispersion (1.0%, w/w protein in 0.1 M phosphate buffer, pH 7.0) at 22,000 rpm for 2 min in a high-speed homogenizer; Ultra Turrax T25 basic (IKA Works, Inc., Wilmington, NC, USA). The emulsion (10 µL) was diluted in 5 mL 0.1 M phosphate buffer (pH 7.0) containing 0.1% sodium deodecyl sulfate (SDS). Absorbance of the diluted emulsion was measured at 500 nm by Spectronic 1200 spectrophotometer (Bausch and Lomb, Rochester, NY, USA). EAI (m^2/g) was calculated as follows:

$$T = (2.303 \times A) / l \qquad (2)$$

$$\text{EAI } (m^2/g) = (2 \times T \times D) / (\varphi \times c \times 10{,}000) \qquad (3)$$

where T is the turbidity, A is the absorbance at 500 nm, l is the path length of the cuvette (1 cm), D is the dilution factor, φ is the volumetric fraction of oil, c is the weight of protein per unit volume of aqueous phase before emulsification was formed (g/mL) and 10,000 is the correction factor for square meters.

Emulsion stability indexes (ESI) were determined in emulsions prepared with 1.0 and 3.0% (w/w) protein stock solutions. Turbidities of emulsions were measured at 0 h and after storage for 24 h at 4 °C. The ESI was calculated as follows:

$$\text{ESI } (h) = (T \times \Delta t) / \Delta T \qquad (4)$$

where T is the turbidity value at 0 h, ΔT is the change in turbidity during the storage period, and Δt is the time interval.

5.3.10. Statistical analysis

Statistical analysis was performed using MINITAB (version 16). Differences between means ($p < 0.05$) were determined by analysis of variance using the general linear models and least square means procedure. All tests were done in triplicates.

5.4. Results and discussion

5.4.1. Particle size and distribution

Table 5.1 shows average particle sizes and polydispersity indexes of non-texturized WPC (control) and TWPCs extruded at different temperatures dispersed in water. The average particle size of non-texturized WPC was 0.310 μm with a polydispersity index of 0.193. The particle sizes of TWPCs extruded at 50 and 70 °C were comparable to that of control. A considerable increase in particle size (0.767 μm) was observed as extrusion temperature increased from 50 to 90 °C, reflecting the increased protein denaturation and aggregation at high temperature. Similarly, aggregates size increased considerably when WPC and β-lg were heated at high temperatures (75–110 °C) under both neutral and acidic pH. This was attributed to an increase in the rate of protein denaturation and aggregation at high temperatures (Durand, Gimel, & Nicolai, 2002; Simmons, Jayaraman, & Fryer, 2007; Spiegel & Huss, 2002).

The polydispersity indices also increased as extrusion temperature was increased, exhibiting a value of 0.196 in TWPC-50 and of 0.327 in TWPC-90. The higher polydispersity indexes indicated a broadening of particle size distributions. The particle size of the TWPC-90 was significantly smaller than those reported in literature (Dissanayake & Vasiljevic, 2009; Dissanayake, Kelly, & Vasilkevis, 2010; Resch, Daubert, & Foegeding, 2004). WP aggregate sizes of > 10 μm were obtained when WP was heated at 80 °C for 1 h at pH 3.0 (Dissanayake *et al.*, 2010). The smaller size of particles observed in the present study could be attributed to the shear and low-acidity (pH 3.0) imposed by the RSCFX process. Similarly, Spiegel and Huss (2002) observed smaller WP aggregates after heating (80 °C) at pH 4.0 than those formed at pH 6.7. The high stability of WP particularly β-lg at acidic pH might have retarded heat-induced denaturation and aggregation due to inhibition of thiol group activation and repulsive intermolecular electrostatic interactions between proteins at low pH (de la Fuente *et al.*, 2002; deWit, 1990; Hoffmann & van Mil, 1997; Shimada & Cheftel., 1989).

Table 5.1. Particle size, polydispersity index, and free sulfhydryl (SH) group content of control and WPC extruded at different temperatures[1].

Samples	Average particle size (μm)	Polydispersity index	Free SH group (μmol SH/g protein)
Non-texturized WPC[2]	0.310 ± 0.004^{b}	0.193 ± 0.019^{b}	24.96 ± 0.96^{a}
TWPC-50	0.308 ± 0.018^{b}	0.196 ± 0.019^{b}	23.83 ± 0.47^{b}
TWPC-70	0.306 ± 0.019^{b}	0.211 ± 0.017^{b}	23.76 ± 0.60^{b}
TWPC-90	0.767 ± 0.104^{a}	0.327 ± 0.024^{a}	22.92 ± 1.27^{b}

[1] Values are means ± standard deviation. Means in the same column followed by the same letter are not significantly different ($p<0.05$)

[2] Non-texturized WPC (control) also contains the added salts, similar to extruded samples.

Simmons et al. (2007) and Spiegel (1999) reported the formation of two types of protien aggregates during heating in the presence of shear, depending on heating temperature. The aggregates formed at 75 °C were soft, small, and weakly bonded, due to weak Van der Waals bonding, whereas the aggregates formed at high temperatures (80–120 °C) were rigid and dense. It is assumed that the TWPC-50 and TWPC-70 formed soft-textured aggregates, while the TWPC-90 formed rigid aggregates. The shear used in the RSCFX process could have disintegrated the aggregates, particularly in TWPC-50 and TWPC-70. It has been reported that the final size of aggregates depends on the balance between aggregate strength, particle collision, and breakage (Queguiner, Dumay, Salou-Cavalier, & Cheftel, 1992). Therefore, the combined effects of acidity and low temperature used in the RSCFX process contributed to the formation of aggregates that would easily disintegrate by the shear friction of the extruder, which resulted in a non-significant difference among the final aggregate size of the TWPC-50, TWPC-70, and non-texturized WPC.

5.4.2. Free sulfhydryl groups

The total free sulfhydryl (SH) groups of WPC and TWPCs are shown in Table 5.1. Total free sulfhydryl (SH) group content in the soluble fraction of non-texturized WPC was 24.96 μmol/ g protein; this was reduced by about 10% to between 22.92 and 23.83 μmol/ g protein after RSCFX. Similarly, several authors have reported that the number of SH groups in WP extrudates (Queguiner et al., 1992) and gels (Lupano, Renzi, & Romera, 1996; Monahan, German, & Kinsella, 1995) formed under acidic conditions

remained identical with the unheated controls. This has been attributed to minimal degradation of thiol groups through sulfhydryl/disulfide interchange reactions in acidic pH range (Shimade et al., 1988; de la Fuente et al., 2002). Therefore, the low pH conditions in the extrusion process diminished the likelihood of TWPC forming new disulfide bonds.

5.4.3. Surface hydrophobicity

The changes in aromatic and aliphatic surface hydrophobicity (S_o) of proteins, as measured by ANS and CPA probes, respectively, are shown in Figure 5.2. Opposite trends were observed for aromatic and aliphatic hydrophobicity with respect to extrusion temperature. Increasing extrusion temperatures increased the accessibility of ANS binding sites, as indicated by enhanced aromatic hydrophobicity. A significantly higher value was obtained for TWPC-90 (377 ± 26) than for TWC-50 and TWC-70. In contrast, RSCFX significantly decreased the aliphatic hydrophobicity from 3691 ± 552 in non-texturized WPC to 1617 ± 240 in TWPC-90. Other studies have also shown that aromatic hydrophobicity of β-lg and bovine serum albumin (BSA) increased on heating from 25 to 85 °C (Cairoli, Iametti, & Bonomi, 1994; Nakai, 1983), while aliphatic hydrophobicity has been reported to decrease with heat treatment (Alizadeh-Pasdar & Li-Chan, 2000; Voutsins, Cheung, & Nakai, 1983). The non-significant changes in aromatic and aliphatic hydrophobicity of TWPC-50 and TWPC-70 with that of non-texturized WPC, implies that only minimal protein denaturation occurred, attributed to greater stability of WP during the RSCFX process in acidic pH. Thus, higher temperature was

required to denature the WP. A higher denaturation temperature has been reported for acidified WP, ranging from 77 to 89 °C (deWit, 1990; Picone, Takeuchi, & Cunha, 2011).

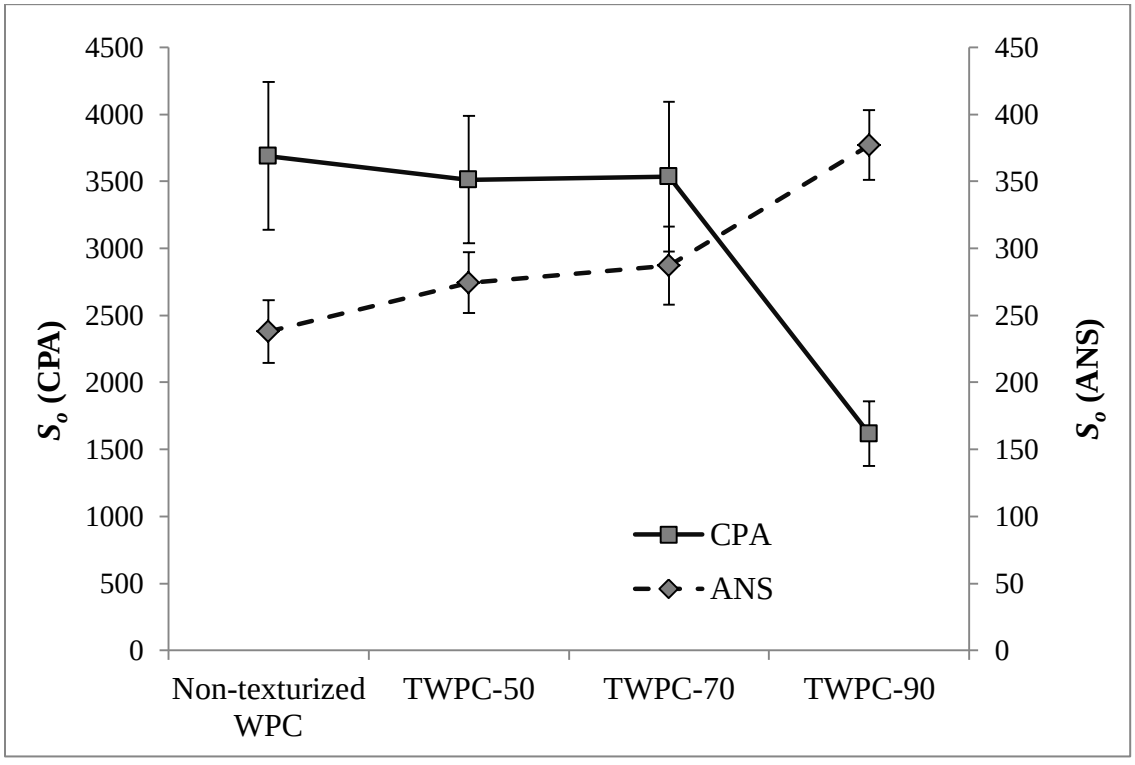

Figure 5.2. Surface hydrophobicity index (S_o) of non-texturized WPC and TWPCs measured using ANS and CPA probes for aromatic and aliphatic hydrophobicity, respectively.

The fact that ANS-binding increased and CPA-binding decreased in all extruded samples implies that the extrusion process rearranged the protein conformations, exposing more aromatic amino acids that were initially concealed in the globular protein (Iametti, Cairoli, De Gregori, & Bonomi, 1995). The greater aromatic hydrophobicity in TWPC-90 indicated extensive protein unfolding at the higher temperature. Unlike aromatic hydrophobic residues, the aliphatic residues are smaller, less hydrophobic, and often found at the surface and interior of proteins (Akita & Nakai, 1990). This explains the high aliphatic hydrophobicity, thus higher protein solubility in water observed in the non-texturized WPC, as well as in samples extruded at a lower temperature. Reduction in aliphatic hydrophobicity at high extrusion temperatures may be due to the burial of aliphatic residues caused by greater intramolecular interactions (Yang, Powers, Clark, Dunker, & Swanson 2002). The present data shows that aromatic hydrophobicity data correlates well with data on particle size. Greater aromatic hydrophobicity resulted in larger particles, confirming the role of extensive exposed hydrophobic groups to the formation of large protein aggregates in TWPC-90.

5.4.4. Protein solubility

Solubility is a key indicator of protein functionality. In this study, samples were dissolved in buffers that disrupt certain types of molecular bonds; ionic and hydrogen bonds (EDTA), hydrogen bonds and hydrophobic interactions (urea and SDS), and disulfide bonds (DTT) (Lee, Morr, & Ha, 1996; Lupano *et al*., 1996). Figure 5.3 illustrates the solubility of the samples in various buffers. Non-texturized WPC showed a

high solubility in deionized water (85.48%) and solubility decreased as a function of increasing extrusion temperature. Both TWPC-50 and TWPC-70 when dissolved in water formed opaque, non-sedimenting dispersions with slightly lower solubilities (79.1% and 77.7%, respectively). On the other hand, a considerable decrease in solubility (24.5%) was observed for TWPC-90, indicating extensive protein denaturation and aggregation. The aggregation of proteins during high temperature extrusion has also been reported by others as a cause for decreased WP solubility (Queguiner et al., 1992). The slight reduction in water solubility observed for both TWPC-50 and TWPC-70 may be due to acid- or heat-induced denaturation of heat-liable proteins of WPC. At pH 3.0, both α-la and BSA are reported to unfold at 42–50 °C, resulting in solubility loss (Paulsson, Hegg, & Castberg, 1985), partly due to the loss of calcium ions that stabilize the α-la molecule (Bernal & Jelen, 1984; deWit & Klarenbeek, 1984). However, it has been reported that the non-covalent interactions induced in β-lg and α-la on heating below 70 °C in the acidic pH range are reversible on cooling (Li-Chan, 1983). In general, proteins unfold reversibly in moderate heat and denature irreversibly to form insoluble aggregates at high temperature (de la Fuente et al., 2002). Therefore, it can be assumed that the slight solubility loss observed in TWPC-50 and TWPC-70 was largely due to acid-induced denaturation.

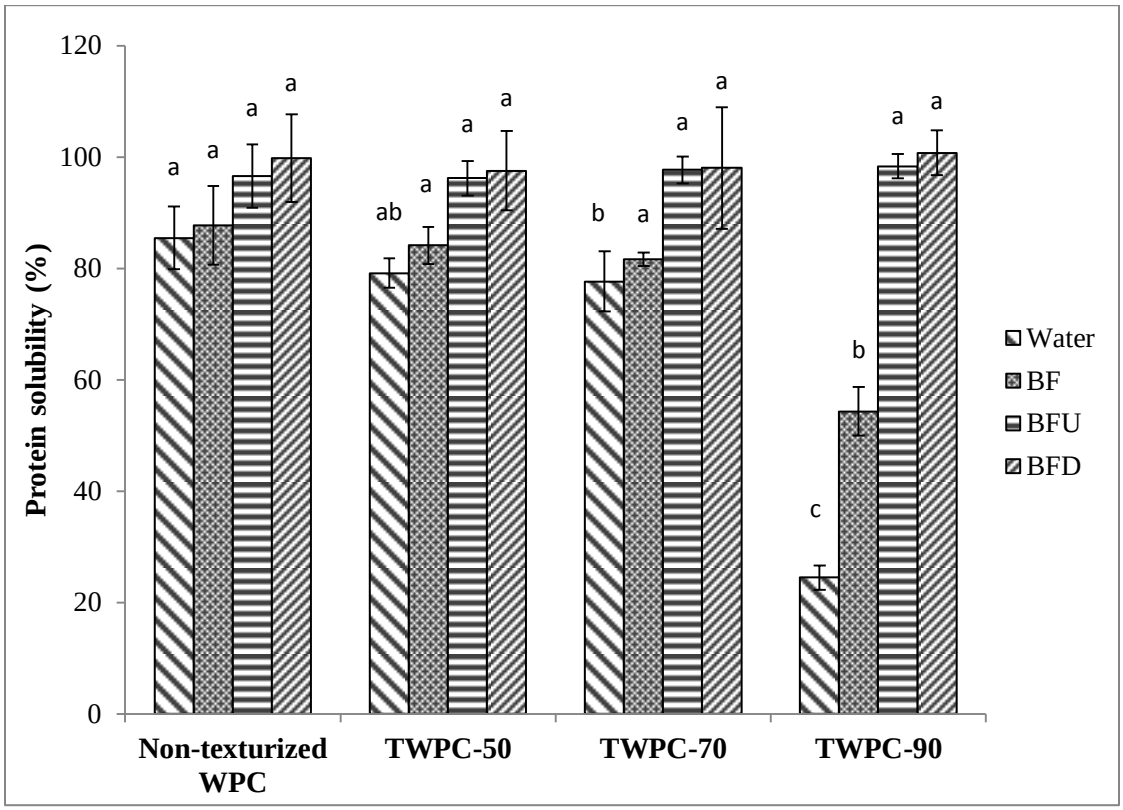

Figure 5.3. Solubility of non-texturized WPC and TWPCs in water and selected buffers. BF: buffer containing 0.086 M Tris, 0.09 M glycine, and 4 mM Na2EDTA, pH 8.0; BFU: BF buffer containing 8 M urea and 0.5% SDS; BFD: BF buffer containing 8 M urea, 0.5% SDS, and 10 mM dithiothreitol (DTT). Means with the same letter are not-significantly different ($p<0.05$).

In TWPC-90, the proteins were most likely extensively denatured, concomitant with the greater exposure of aromatic hydrophobic residues causing a higher degree of protein–protein interactions over protein–water interactions. This behavior therefore, favors protein aggregation and causes loss in solubility. This finding is in agreement with previous literatures that stated aromatic hydrophobicity of β-lg has been correlated with protein insolubility (Hayakawa & Nakai, 1985; Nakai, 1983). There is a negative correlation between aliphatic hydrophobicity and insolubility with increasing temperatures (70–95 °C). Proteins with high surface hydrophobicity showed little water solubility owing to their natural tendency to shield non-polar residues from the aqueous environment by forming aggregates through hydrophobic interactions (Alijadeff, 2008). The solubility of TWPC observed in the present study was higher than the values reported for WP extrudates obtained by Onwulata, Konstance, Cooke, and Farrell (2003) and Qi and Onwulata (2011). The authors demonstrated an approximately 65% reduction in solubility of WPI extruded at 75 °C and only 1–3% protein remained soluble at 100 °C. The minimal loss of solubility in the present study may have been attributed to the greater thermostability of β-lg at acidic pH (pH $\leq$ 3.0) imposed during the RSCFX process.

Solubilization of the protein in standard buffer BF marked a significant increase for TWPC-90 (122% increase). This resulted in a solubility index value of 54.3%. Other samples showed a 2.64% to 6.3% increase, resulting in solubility values ranging from 84.1% to 87.7%. The increase in solubility indicated the presence of non-covalent interactions that are electrostatic in nature, which stabilized the native WP and TWPC aggregates (Cavallieri, Costa-Netto, Menossi, & da Cunha, 2007). The solubility of all

the samples increased in buffer that contained urea and SDS (BFU). TWPC-90 showed the greatest increase with a solubility of 98.3% and fell within the statistically insignificant range of 96.2–98.3% shown by other samples, suggesting that other non-covalent interactions such as hydrogen bonds and hydrophobic interactions contributed in stabilizing the protein aggregates. Similar findings were reported by several authors (Mudgal, Daubert, Clare, & Foegeding, 2011; Shimada & Cheftel, 1989) for WP system in acid-pH range.

For all samples in buffer with reducing agent, DTT (BFD), solubility increased to 97.53 to 100.76%. The non-significant difference in protein solubility in BFU and BFD buffers confirms that disulfide bonds were not significantly involved in stabilizing the structures of acidic TWPC aggregates. These findings are in agreement with studies reported in the literature. Heating WPC in pH 3.35–4.60 and at ≥ 80 °C was found to result in aggregates that were only partly disulfide-linked (Clare, Lillard, Ramsey, Amato, & Daubert, 2007; Li-Chan, 1983). Queguiner et al. (1992) also demonstrated that the reactivity of SH group of microparticulated WPI decreased significantly in acidic conditions (pH 2.5–3.5) attributed to high stability of thiol group in acid conditions. As expected, RCFSX conducted at low and intermediate temperatures of 50–70 °C produced protein aggregates with higher water solubility; high temperatures of 90 °C resulted in aggregates with reduced solubility. The protein aggregates were predominantly stabilized by non-covalent interactions and that disulfide bonds played only a minor role.

5.4.5. Steady shear properties

Apparent viscosity of water dispersions (22–26%, w/w) of non-texturized WPC and TWPCs was shown in Figure 5.4. Non-texturized WPC had the lowest viscosity, ranging from 0.010 to 0.020 Pa.s. Increasing the concentration of protein from 20–26% (w/w) did not affect viscosity. The viscosity-shear rate profile (Figure 5.5) indicates that non-texturized WPC has Newtonian-like behavior, with a flow index ranging from 0.917 to 0.957. When reconstituted in water at room temperature, TWPC exhibited enhanced thickening capacity. Extrusion temperature played an important role in determining the final viscosity of TWPCs. The texturized samples exhibited 114 to 398-fold increases in viscosity compared to non-texturized WPC with TWPC-90 was the highest at all concentrations. Both TWPC-50 and TWPC-70 formed viscous, homogenous pastes at 20% (w/w) protein with viscosities of 1.10 and 1.16 Pa.s (at shear rate of 25 s^{-1}), respectively (Figure 5.6), whereas TWPC-90 exhibited a viscosity of 2.838 Pa.s. In comparison to TWPC-50 and TWPC-70, the TWPC-90 showed 2.1 to 2.8-fold increases. Several studies have reported similar observations. Resch *et al*. (2005) demonstrated a steady increase in viscosity of reconstituted derivatized WPI powders (10%, w/v) produced at pH 3.35 by increasing temperature from 75 to 90 °C. In an earlier study, Hongsprabhas and Barbut (1996) showed that a stronger calcium-induced cold-set gel was obtained when WPI was pre-heated to ≥ 80 °C. This is due to enhanced inter-molecular cross-linking facilitated by pre-heating. In this study, cold-set gel was obtainable by RECFX process even at 50 °C. In contrast, Barbut and Foegeding (1993) reported preheating WPI at neutral pH to a minimum of ≥ 70 °C for 10 min to achieve optimum $CaCl_2$ sensitivity to form cold-set gels. A minimum concentration of

approximately 24% (w/w) for TWPC-70 and 25% (w/w) for TWPC-50 were needed to form cold-set gels having viscosity comparable to that of 20% (w/w) TWPC-90.

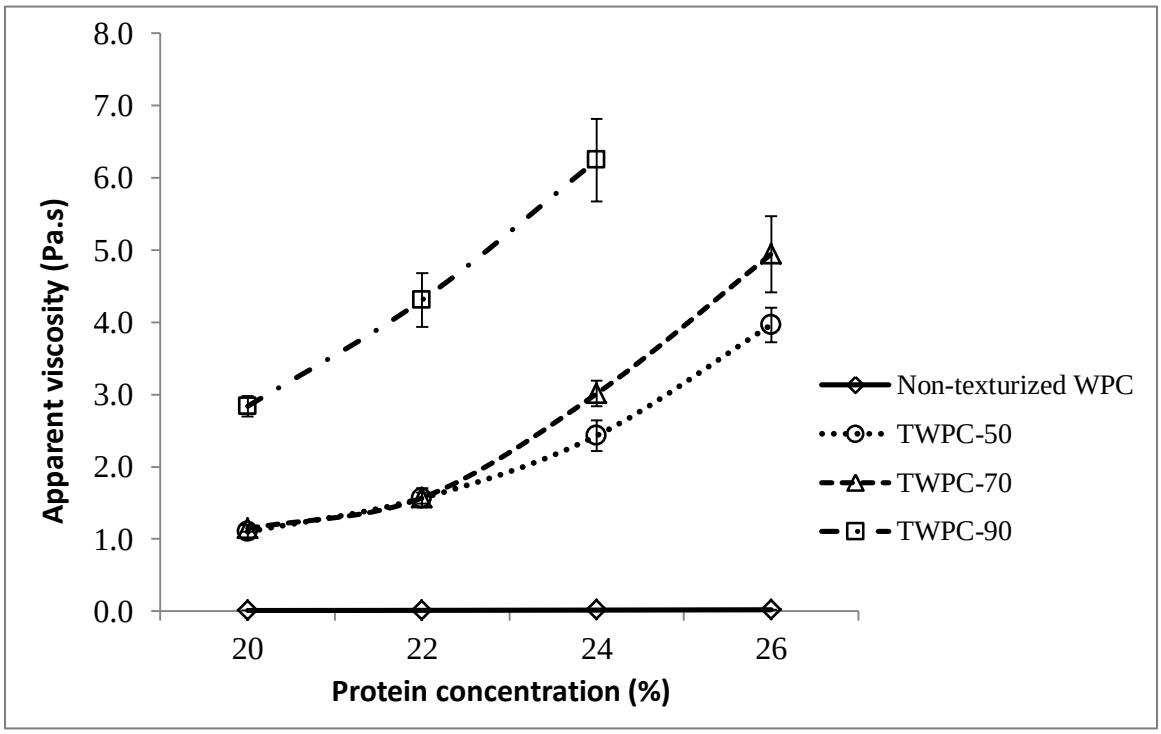

Figure 5.4. Changes in apparent viscosity (at 25 s^{-1}) of non-texturized WPC and TWPCs at various concentrations.

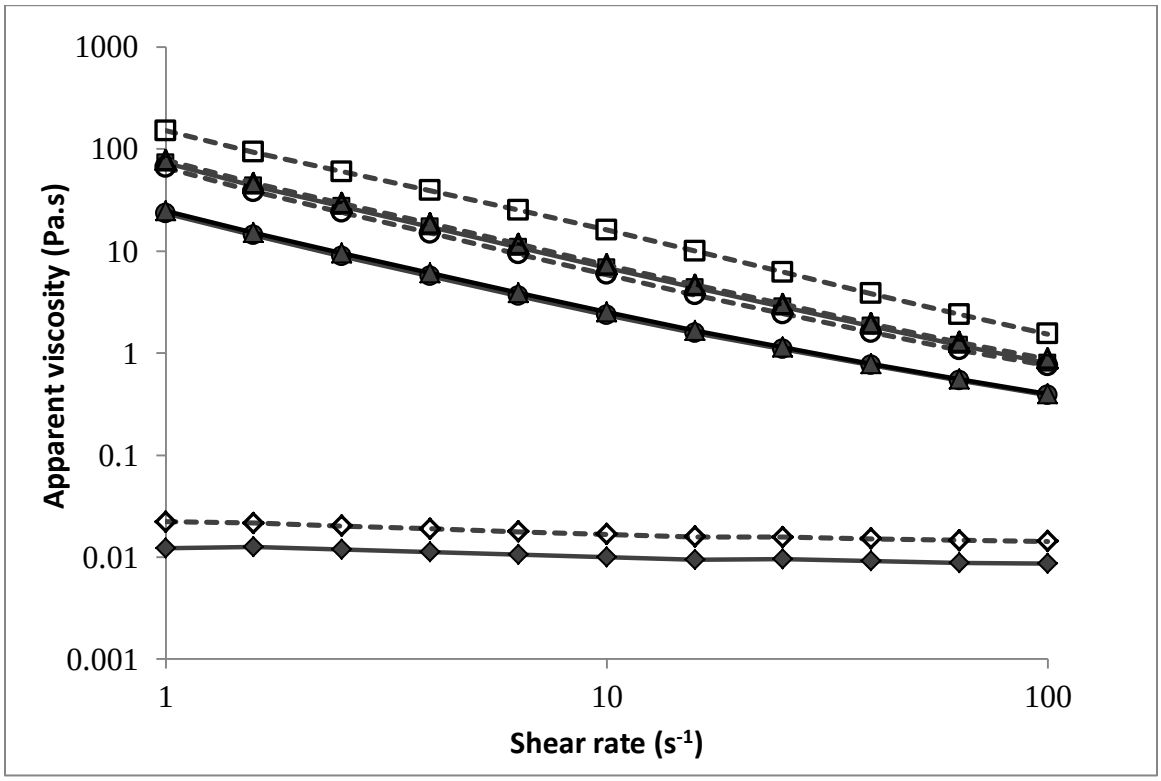

Figure 5.5. Flow behavior profile of non-texturized WPC dispersion (◊) and TWPC cold-set gels; TWPC-50 (○), TWPC-70 (Δ), and TWPC-90 (□) at 20% (filled symbols) and 24% (w/w) (empty symbols).

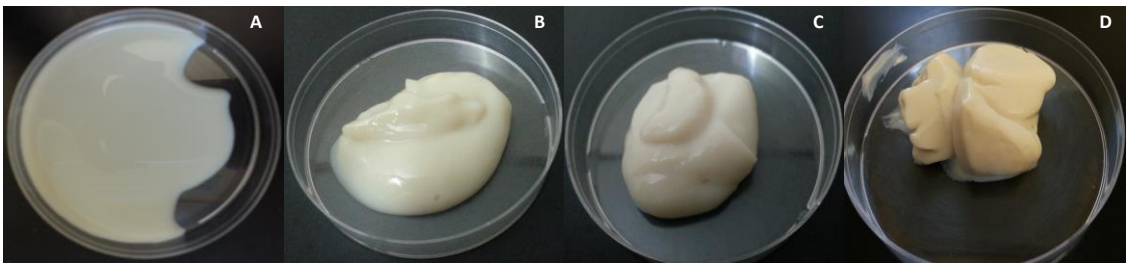

Figure 5.6. Non-texturized WPC and TWPCs reconstituted in water at 20% (w/w) protein. (A) non-texturized WPC, (B) TWPC-50, (C) TWPC-70, and (D) TWPC-90.

Table 5.2. Power Law parameters[1] representing apparent viscosity (η_a), consistency index (K) and flow behavior index (n) of WPC and TWPC dispersions and gels.

Samples	Protein concentration (%, w/w)	Power law parameters		
		η_a (Pa.s)[2]	K (Pa.s^n)	n
Non-texturized WPC	20	0.010^{g}	0.01^{g}	0.917^{a}
	22	0.012^{g}	0.02^{g}	0.922^{a}
	24	0.016^{g}	0.02^{g}	0.921^{a}
	26	0.020^{g}	0.02^{g}	0.957^{a}
TWPC-50	20	1.097^{f}	20.46^{f}	0.112^{b}
	22	1.565^{f}	32.25^{f}	0.078^{b}
	24	2.431^{e}	58.67^{e}	0.027^{c}
	26	3.966^{c}	101.40^{c}	0.027^{c}
TWPC-70	20	1.145^{f}	22.17^{f}	0.096^{b}
	22	1.570^{f}	32.04^{f}	0.081^{b}
	24	3.013^{d}	72.60^{d}	0.022^{c}
	26	4.944^{b}	125.55^{b}	0.007^{c}
TWPC-90	20	2.838^{de}	68.27^{de}	0.025^{c}
	22	4.309^{c}	106.97^{c}	0.015^{c}
	24	6.245^{a}	152.68^{a}	0.006^{c}
	26	NA	NA	NA

[1] Means in the same column followed by the same letter are not significantly different ($p < 0.05$)

[2] The apparent viscosity was compared at 25 s^{-1}; NA: Test was not performed because sample was too thick to stir.

Like polysaccharide-based thickening agents, all of the samples exhibited pseudoplastic or shear thinning behavior, evidenced by decreasing viscosity with increased shear rate. Table 5.2 shows that all the extruded samples had a very low flow behavior index (*n*), ranging from 0.006 to 0.112, the lowest for TWPC-90. Flow behavior index decreased as TWPC concentration increased. On the other hand, consistency index exhibited a trend similar to apparent viscosity. Samples showed increasing consistency index in the order of; non-texturized WPC > TWPC-50 > TWPC-70 > TWPC-90.

Viscosity is influenced by the shape, size, and polydispersity of protein aggregates, hydrodynamic volume, interactions among the aggregates, protein–solvent interactions, and the molecular flexibility of proteins in their hydrated state (Mudgal *et al*., 2011; Vardhanabhuti & Foegeding, 1999). Differences in final viscosities of the TWPCs may be attributable to the degree of protein denaturation and aggregation. It is assumed that the proteins in TWPC-50 and TWPC-70 are partially denatured and those in TWPC-90 are extensively denatured. Therefore, the higher viscosity of TWPC-90 indicates the existence of comparatively greater inter-molecular cross-linking. This can be attributed to the exposure of a greater number of reactive groups that facilitated non-covalent protein–protein interaction, as alluded by the solubility values in different types of solvents shown earlier. In addition, enhanced protein–water interaction enabled the entrapment of a greater number of water molecules within the network, enhancing the water-binding capacity of the cold-set gels. Similarly, higher pre-heating temperatures produced gels with greater rigidity (Hongsprabhas & Barbut, 1996; Vandhanabhuti & Foegeding, 1999). It was proposed that heating at a lower temperature (70 °C) resulted in

the formation of β-lg–α-la dimers, whereas, a higher temperature (90 °C) resulted in a greater number of high molecular weight polymers (Zhu & Damodaran, 1994). The presence of polymerized WPI was reportedly able to enhance water-holding capacity of the gels due to their greater ability to entrap water than the individual WP molecules (Firebaugh, 2004; Vardhanabhuti et al., 2001). Thus, the larger aggregates in TWPC-90 reported above, may lead to greater hydrodynamic volumes, resulting in reduced minimum concentrations required for cold-set gelation.

Although the solubility and particle size of TWPC-50 and TWPC-70 were comparable to non-texturized WPC, the viscosity of the latter was significantly higher. It is speculated that other factors, such as surface hydrophobicity and the presence of insoluble aggregates, influence the increase in viscosity of TWPC. The increase in exposed aromatic surface hydrophobicity of TWPCs might also enhance intermolecular hydrophobic interactions. The thickening and gelation properties of WPC, β-lg, and albumen have been reported to correlate positively with protein hydrophobicity (Cheng, 2001; Nakai, 1983; Voutsinas et al., 1983). It has been shown that gels formed mainly by hydrophobic bonds were able to retain higher amount of bound water (Clare et al., 2007; Hongsprabhas & Barbut, 1997; Sorgentini et al., 1991; Petit et al., 2012). In addition, the presence of insoluble protein aggregates has been correlated with improved strength of WPC (Puyol et al., 1999) and soy protein gels (SPI) (Sorgentini et al., 1995). This has been attributed to the greater water-imbibing capacity of the aggregates as compared with the soluble fraction (Sorgentini et al., 1991, 1995). These reports support our findings, in which TWPC-90, consisting of large amounts of insoluble aggregated protein with high surface hydrophobicity, exhibited high viscosity with improved gelation properties.

5.4.6. Thermal stability

Figure 5.7 shows the changes in storage modulus (G') and loss modulus (G") of non-texturized WPC and TWPC dispersions/gels when heated from 25 to 95 °C. The values of G' for all TWPCs were greater than G" prior to heating, indicating a solid-like behavior. On the other hand, the moduli of the non-texturized WPC fluctuated below 80 °C, and on heating beyond that temperature, G' surpassed G". On continued heating, both of the moduli were further increased, and at about 79 °C a steep increase in G' was observed for all samples except TWPC-90. This sharp increase in G' marked the beginning of network formation that occurred after 36 min of heating. The TWPC-50 and TWPC-70 also exhibited a steeper increase in G' and G" compared with the non-texturized WPC. Our findings correlate well with Picone et al. (2011) and Dissanayake et al. (2012) who reported that both native β-lg and heat-denatured WPI at pH 3.0–3.35 underwent gelation at 80 °C. In contrast, several authors (McClements & Kegoh, 1995; Vardhanabhuti et al., 2001) have shown that WPI containing WP polymers or preheated WPI formed heat-induced gels at lower temperatures (~29 to 72 °C) compared to their corresponding native WPI (~70 to 76 °C). In the present study, comparable gelation temperature and time observed for both TWPCs and non-texturized WPC may be due to higher stability of the acidic texturized WPC. At the end of the heating period, the values of G' for both TWPC-50 and TWPC-70 gels surpassed TWPC-90 by 2.4-fold. Both samples exhibited a tremendous increase in G', with final values ranged from 14672–14920 Pa, forming the strongest gels. On the other hand, the G' of TWPC-90 was fairly constant throughout the heating process, exhibiting a final value of 6198 Pa. The non-texturized WPC showed the lowest final G' values (1616 Pa), implying the weakest gel.

Higher G' is an indication of the existence of comparatively higher intermolecular interactions in the viscoelastic system.

It has been reported that heat-set WP gels formed at acidic pH were relatively brittle and weak (Havea, Watkinson, & Kuhn-Sherlock, 2009; Hudson et al., 2000; Morr & Ha, 1993) due to relatively weak linkages—attributable to inhibition of thiol group activity and intermolecular repulsive electrostatic interactions (Morr & Ha, 1993; Spiegel & Huss, 2002). In contrast, the acidic TWPC gels were significantly stronger. The difference in the behavior can be explained by the magnitude of WP denaturation and state of protein constituents. The TWPC-50 and TWPC-70 were partially denatured, thus consisted of both native and polymerized protein molecules. The presence of exposed reactive groups increased the sensitivity of the denatured proteins for intermolecular interactions during heating, as indicated by a steep increase in moduli, leading to faster network formation. A significant amount of remaining native protein has been reported following heating of WP at $\leq$ 70 °C with only 35–50% WP denatured after 90 min of heating (Dannenberg & Kessler, 1988; Hofmann, Sala, Olieman, & De Kruif, 1997). It was assumed that a high amount of native WP remained in the TWPC-50 and TWPC-70. The increase of moduli of the TWPCs is attributable to the formation of additional networks following unfolding and aggregation of native proteins remaining in the samples. This resulted in a comparatively greater extent of attractive intermolecular cross-linking in the TWPC system. This behavior implied that second gelation is taking place in TWPC-50 and TWPC-70. The present results suggest that the degree of protein–protein interactions can be increased and enhanced by reheating.

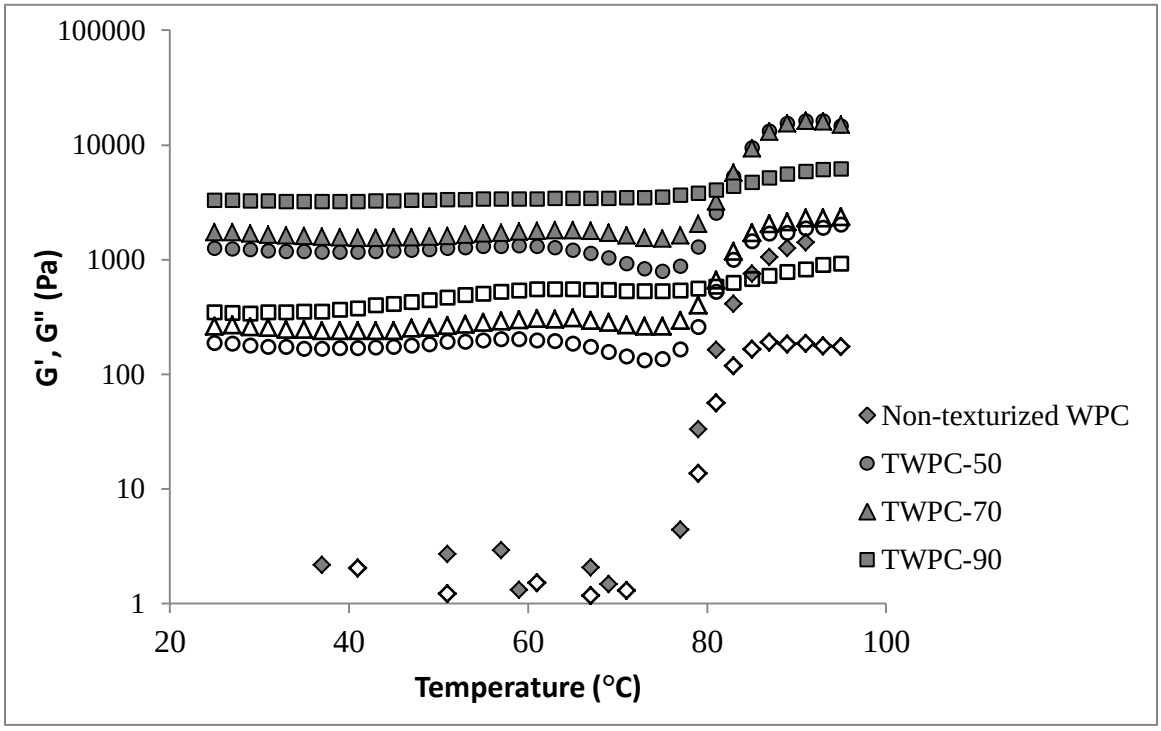

Figure 5.7. Changes in G' (storage modulus) and G" (loss modulus) of protein in water dispersions/gels during heating from 25 to 95 °C at 2 °C/min. Filled symbols represent G' and empty symbols represent G".

The findings are in agreement with Barbut and Drake (1997), in which heating of cold-set WPI gels at 80 °C for 30 min significantly increased the complex modulus by a factor of 2.5 to 8.0. Vardhanabhuti et al. (2001) studies the effects of ratios of native WPI and WP polymers on the increase of G' during heating at 80 °C. Protein solutions that contain only native WPI had the lowest G' values, whereas solutions with higher

amounts of WP polymers had higher final G' values. The authors proposed that the heat-induced gelation of monomers and cold gelation of aggregates occur concurrently in a system containing a mixture of native and aggregated WP. It has been demonstrated that differences in the state of protein could be directly related to the rate of gelation and the final gel structure (Hongsprabhas & Barbut, 1998; Khun, Cavallieri, & Cunha, 2010; McClements & Keogh, 1995).

In TWPC-90, the formation of a strong, heat-induced gel was unlikely due to the almost complete disappearance of native WP, leading to the unavailability of reactive sites for additional cross-linking. Similarly, Hudson and Daubert (2002) and Resch and Daubert (2002) found that derivatized WPC and WPI were relatively equal in viscosity when heated to high temperature (75-90 ˚C) to those before heating. The derivatized WPI contained only ~5.0% of native proteins (Resch & Daubert, 2002), which conforms that the majority of proteins in the derivatized powders were denatured (Hudson et al., 2000). Completely denatured proteins were incapable of additional unfolding and interaction during heating (Dissanayake et al., 2010, 2012; Hudson & Daubert, 2002). Therefore, the slight increase in G' of TWPC-90 can be proposed as a function of additional unfolding and denaturing of remaining native or renatured WPs.

Tan δ, a measure of viscoelasticity of a gel was less that 0.2 for all TWPC samples throughout the heating course. A slightly higher tan δ values (0.14-0.16) were obtained for TWPCs at the end of the heating course, compared with non-texturized WPC (tan $\delta = 0.11$). This result implies that native WPC and TWPC formed gels with different molecular properties, in which the former produced a slightly elastic gel. The difference in elasticity of WPC and TWPC gels may be due to different intermolecular interactions

involved in the formation of the gels. Acidic TWPC gels were formed mainly by non-covalent interactions (hydrophobic and hydrogen bonds), whereas WPC gel formed at near neutral pH (6.5) was stabilized by both covalent (disulfide) and non-covalent bonds. It has been reported that the high rigidity of heat-induced WP gels formed at pH 6.4–6.6 was due to interactions of thiol groups (deWit, 1989).

These results show that RSCFX-based texturization resulted in WPC that can form heat and cold-set gels with molecular and physical characteristics different from those of untreated control. TWPC obtained at low and high temperatures can be advantageously used in different product applications. Heating the cold-set gels made from TWPC-50 and TWPC-70 enhanced their strength, suggesting the potential use of secondary heating to enhance gel characteristics, while the excellent temperature stability of TWPC-90 could be an advantage in certain product applications requiring high thermal stability.

5.4.7. Emulsifying properties

The emulsifying properties of TWPC, expressed as the emulsifying activity index (EAI) and emulsion stability index (ESI) are presented in Table 5.3. The EAI values of all TWPCs were not significantly different from non-texturized WPC, regardless of extrusion temperature and ranged from 37.50 to 39.78 m^2/g. Dissanayake *et al*. (2012) and Firebaugh and Daubert (2005) demonstrated that heat-treated, acidified (pH 3.0) WP displayed an emulsifying capacity similar to native controls. However, since the non-texturized WPC contains added salts, commercial WPC was evaluated to determine the impact of salt on EAI. The EAI of commercial WPC was lower (35.74 m^2/g), implying that the presence of salts in non-texturized WPC might improve its EAI. It can be concluded that RSCFX significantly improves EAI in contrast to commercial WPC. Contradictory results have been reported for the emulsifying capacity of heat-denatured WP. Studies on WPI blend consisted of different ratios of denatured proteins (80 °C for 15 min), indicated that a higher proportion of denatured WPI improves EAI (Britten et al., 1994). In contrast, Fachin and Viotto (2005) and Resch and Daubert (2002) proposed that heat treatment reduces the efficiency of emulsification.

Table 5.3. Emulsifying activity index (EAI) and emulsion stability index (ESI) of emulsions prepared with control and texturized WPC[1].

Samples	EAI (m^2/g)	ESI (h)	
		1% protein	3% protein
Non-texturized WPC	$39.74^{a} \pm 2.15$	$34.15^{c} \pm 2.33$	$41.26^{c} \pm 2.02$
TWPC-50	$37.50^{a} \pm 2.40$	$34.29^{c} \pm 1.39$	$52.59^{b} \pm 4.47$
TWPC-70	$39.78^{a} \pm 2.35$	$36.24^{b} \pm 0.84$	$54.48^{b} \pm 5.75$
TWPC-90	$38.64^{a} \pm 2.50$	$38.15^{a} \pm 1.25$	$233.37^{a} \pm 55.27$

[1] Values are means ± standard deviation. Means in the same column followed by the same letter are not-significantly different ($p<0.05$)

An increase in protein surface hydrophobicity has been suggested as one of the factors that promotes the emulsifying properties of WP (Monahan, McClements, & Kinsella, 1993; Nakai, 1983; Voutsinas et al., 1983). The increase in exposed aromatic residues might have improved the interfacial properties of the TWPCs attributed to the increased affinity of the proteins towards the oil-water interface. Although TWPC-90 has greater aromatic surface hydrophobicity, the balancing effect of low protein solubility produces a slightly lower EAI. Moderate-heat treatment of WPC and WPI has been shown to improve their emulsifying properties, whereas heating that causes excessive protein aggregation results in a decrease in their emulsifying properties (Britten et al., 1994; Dybowska, 2011). As solubility was progressively decreased by heat denaturation, it became an increasingly significant factor governing emulsification in addition to hydrophobicity (Nakai, 1983; Voutsinas et al., 1983). Therefore, a certain degree of protein denaturation and aggregation that result in optimum aromatic surface protein hydrophobicity and good solubility may be required for optimal emulsifying activity of TWPCs.

Emulsion stability was evaluated using ESI at 1% and 3% (w/w) protein. ESI can be regarded as an indicator of WP's ability to retard phase separation (creaming) and coalescence during storage, the main factors in emulsion destabilization (Pearce & Kinsella, 1978). Emulsions prepared with 1% (w/w) TWPC-70 and TWPC-90 exhibited higher stability (36.24 to 38.15 h) in comparison with non-texturized WPC (34.15 h). Increasing protein concentration to 3% (w/w) significantly increased the emulsion stability of all samples. TWPC-90 exhibited the highest increase (503%) with an ESI of

233.37 h, whereas non-texurized WPC had an increase of only 21% with an ESI of 41.26 h. No significant difference was observed between ESI values for TWPC-50 and TWPC-70. Similar behavior was observed by Dybowska (2011) who demonstrated emulsions of improved stability was obtained with WPC that was preheated at higher temperatures (80–90 ˚C) compared to lower preheating temperatures (60–70 ℃). The increase in emulsion stability with increasing extrusion temperature may be attributed to higher continuous-phase viscosity that minimizes the movement of fat droplets, hence retards the creaming process. In addition, the aggregated proteins of the TWPC may have formed a thicker protein membrane, thus providing better stabilizing properties for the fat droplets. Increasing the derivatized WPI in a protein blend was reported to reduce coalescence of emulsion, probably due to the formation of a thicker protein membrane that resists mechanical deformation and coalescence (Britten et al., 1994). In addition, increasing the protein level to 3% not only increases the viscosity of emulsion, but it also provides more protein to coat the fat droplets, thus preventing the occurrence of flocculation or coalescence. Therefore, it is concluded that ESI was predominantly affected by the viscosity of the aqueous phase and the strength of protein–oil and protein–protein interactions at the interface. On the other hand, the EAI was mainly affected by the balance of solubility and surface hydrophobicity.

5.5. Conclusions

RSCFX processing of WPC at 50 and 70 °C caused only a minimal loss of protein solubility, whereas at 90 °C proteins were extensively denatured, causing a considerable increase in aggregates size and a large reduction in solubility. Increasing extrusion temperatures increased the aromatic hydrophobicity and decreased the aliphatic hydrophobicity. Non-covalent interactions were the main contributors in the formation of TWPC aggregates, which showed excellent thickening characteristics, resembling polysaccharide thickeners, at room temperatures. The formation of cold-set gels of TWPC modified at lower extrusion temperatures (50–70 °C) was also possible by utilizing 24 to 25% (w/w) TWPC. Second-stage gelation occurred during heating the cold-set TWPC-50 and TWPC-70 gels, resulting in gels stronger than those of TWPC-90. The presence of partially denatured proteins in TWPC-50 and TWPC-70 created additional intermolecular cross-linking in the gels. TWPC-90 formed cold-set gels with a good temperature stability, which was unaffected by further heating temperatures. The greater exposure of aromatic hydrophobic residues and increased aqueous phase viscosity improved the emulsifying properties of TWPC. The TWPC extruded at different temperatures can be advantageously utilized in various product applications.

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CHAPTER 6

STORAGE AND HEAT STABILITY OF EMULSION GELS MADE FROM REACTIVE SUPERCRITICAL FLUID EXTRUSION (RSCFX) MODIFIED WHEY PROTEIN CONCENTRATE

6.1. Abstract

Whey protein concentrate (WPC) was extruded using RSCFX process to produce texturized WPC at 70 (TWPC-70) and 90 °C (TWPC-90). TWPCs were used to form cold-set emulsion gels containing 40–80% (w/w) oil, and their storage (4 °C for 30 days) and heat (70–90 °C for 20 min) stability were determined. The experimental results revealed that TWPC emulsions had smaller droplet sizes, greater protein loads, and consisted of higher amounts of α-lactalbumin and high molecular-weight protein compared to the non-texturized WPC. The adsorbed proteins of non-texturized WPC and TWPC-70 underwent time-dependent polymerization during storage, but those in TWPC-90 did not. On heating of emulsions, the adsorbed TWPC proteins showed polymerization, while desorption was observed in non-texturized WPC. Possible explanations for the superiority of TWPC-stabilized emulsions are combinations of a stable protein gel matrix that helped to form the continuous phase, greater surface hydrophobicity, and intra-film protein polymerization that conferred strength to the protein interfacial layer. TWPC-90, which contains a higher degree of denatured proteins, offers the best potential to serve as a novel, whey protein-based food emulsifier and stabilizer.

6.2. Introduction

Whey protein (WP) is frequently used as an emulsifier due to its amphiphilic nature, while polymeric protein stabilizes emulsions primarily by a combination of steric and electrostatic interactions (Dickinson, 2001). During emulsion formation, protein rapidly adsorbs at the oil droplet surface, followed by surface denaturation; the protein molecule unfolds, hydrophobic residues reorient towards the oil phase, and the molecule spread to varying extents to form a continuous viscoelastic film (Kinsella, 1984; Phillips, 1981). The effectiveness of protein as an emulsifier depends on the number and type of contacts it makes at the interface. A protein molecule that is flexible and has a high proportion of hydrophobic residues is more effective at reducing interfacial tension because of its ability to create more contacts with the oil surface (Dickinson, 1999; Dickinson & McClements, 1995; Dickinson, Murray & Stainsby, 1988). Although aggregated protein is less efficient as an emulsifier due to reduced surface activity, it shows better stabilizing properties in protecting droplets against creaming (Euston & Hirst, 1999).

The physicochemical properties of emulsions are largely determined by the nature—structure and composition of the adsorbed protein layer (Dickinson, 1997, 2012). The storage and heating of emulsions are known to influence the stability of WP-stabilized emulsions, attributable to changes in the conformational structure of protein molecules at the interfacial layer (Damodaran & Anand, 1997; Das & Kinsella, 1990). During storage of emulsions, time-dependent formation of high molecular-weight polymers have been reported to occur at the interface of droplets in β-lactoglobulin (β-lg) and in β-lg–α-lactalbumin (α-la)-stabilized emulsions. The polymerization of adsorbed

protein via sulfhydryl/disulfide interchange reactions was suggested to be responsible for the increase in viscoelasticity of the protein membrane (Dickinson & Matsumura, 1991; Dickinson, Rolfe & Dalgleish, 1990; McClements, Monahan & Kinsella, 1993a).

In addition to emulsion formation and stabilization, WP is also used to modify the texture, mouthfeel, and overall acceptability of food emulsions (Kinsella, 1984). This is frequently done with the use of heat-treated emulsion gels (Chen & Dickinson, 1998; Yost & Kinsella, 1992). During heat treatment, fat droplets are incorporated into the matrix of heat-set protein gels. This occurs via interactions between adsorbed protein at the interface and those of the gel matrix through a combination of disulfide, hydrophobic, and hydrogen bonds (McClements, Monahan & Kinsella, 1993b; Chen & Dickinson, 1999b). The viscoelasticity of a gel is influenced by oil volume fraction, average droplet size, and the type of interactions between the gel matrix and filler particles (Chen & Dickinson, 1999a; Chen, Dickinson, Langton & Hermansson, 2000). In some cases, heating of emulsion leads to polymerization of both adsorbed proteins and proteins in the continuous phase, which influences the state of aggregation of fat droplets. The changes in stability of emulsion droplets during heating often depend on factors such as temperature and ionic strength. Droplet aggregation is not observed when heating WPI emulsions up to 70 °C, but heating at 75–80 °C leads to fat droplet aggregation, attributable to thermal denaturation of protein that causes inter-droplet interactions. Intra-droplet protein–protein interactions are favored at higher temperatures (> 80 °C) (Monahan, McClements & German, 1996; Demetriades, Coupland & McClements, 1997). Aggregation of fat droplets affects both the stability and rheology of heated emulsions including droplet size, creaming rates, and viscosity (Keowmaneechai &

McClements, 2006; Monahan et al., 1996; Sliwinski, Roubos, Zoet, van Boekel & Wouters, 2003).

WP emulsion gels can also be formed by a cold gelation process, thus increasing their utility in food formulations where heating is undesirable. In this technique, pre-heated WP aggregates are used to prepare oil-in-water emulsion, and the gelation of emulsion is achieved by adding salts (Sok Line, Remondetto & Subirade, 2005) or by acidification (Alting, Hamer, de Kruif & Visschers, 2003; Boutin, Giroux, Paquin & Britten, 2007). Physical interactions and disulfide bonds, respectively, are the predominant forces in the first and second stages of cold-set emulsion gel formation.

The stability of WP emulsions is of great interest to the food industry. It is common for food emulsions to be stored or heated, and therefore the stability and rheological properties of emulsions are highly important. These properties are strongly dependent on the sizes of fat droplets in the dispersed phase, which is controlled by the properties of the protein membranes surrounding the droplets (Dickinson, 2006; Dybowska, 2011). Heat treatment and storage stability of emulsions stabilized by native WPI and β-lg have been studied with respect to the stability of oil droplets and changes in proteins at the interfacial layer (Damodaran & Anand, 1997; Monahan *et al*., 1996). However, what has gone largely unexplored is how rheology and droplet stability of concentrated emulsions is affected by replacing native WP with partially or extensively denatured WP. Therefore, the objective of this study was to investigate the effects of both the type and extent of protein denaturation and the concentration of oil on the stability of concentrated cold-set emulsion gels. The reactive supercritical fluid extrusion process (RSCFX) was used to modify whey protein concentrate (WPC) at different

extrusion temperatures (70 and 90 °C) under acidic conditions (pH 3.0) to obtain texturized WPC (TWPC) with varying degrees of protein denaturation. TWPC that formed cold-set protein gels was used to form cold-set emulsion gels, which were then subjected to long-term storage and thermal treatments. The stability of the emulsion gels was studied by measuring droplet sizes, rheological properties, emulsion microstructures, protein adsorption at the oil-water interface, and electrophoretic analysis of the adsorbed protein.

6.3. Materials and Methods

6.3.1. Materials

Commercial WPC-80 was purchased from Leprino Foods Company (Denver, CO, USA). The composition of the WPC was 81.5% protein (dry basis), 5.5% fat, 6.5% lactose, and less than 3.0% ash. Commercial soybean oil was purchased from a local store.

6.3.2. Texturization of whey protein by RSCFX

A protein blend was prepared by pre-hydrating WPC-80 to 10% (w/w) moisture. 0.6% NaCl and 0.3% $CaCl_2$ (w/w) were added to the pre-hydrated WPC. The premixed protein blend was then preconditioned overnight at room temperature and extruded using a co-rotating twin-screw extruder (Wenger TX-52 Magnum, Sabetha KS, USA) coupled with supercritical carbon dioxide (SC-CO_2) injection system. The extruder, with a L/D

ratio of 28.5:1, was configured for the RSCFX process and SC-CO_2 was injected into the barrel through four valves located at L/D ratio of 24. The protein blends were extruded at two different die-exit temperatures: 70 and 90 °C and labeled as TWPC-70 and TWPC-90, respectively. The extruder was operated at 130 rpm at a feed rate of 35 kg/h. SC-CO_2 (1.5%, w/w of dry feed) was continuously injected into the protein dough at a pressure of 10-15 MPa. An aqueous solution of 15% (w/w) HCl was injected into the mixing zone to obtain extrudate of pH 3.0 and the extrusion was carried out at 60% moisture content. The extrudate was pushed through two die inserts with 1.2 mm diameter circular openings and was dried at 40°C for 16 h to achieve 5–6% moisture content. The dried products were finely ground in a mill with a 1.0-mm sieve (Thomas-Wiley Mill model ED-5, Arthur H. Thomas Co., PA, USA), and stored in air-tight containers at room temperature.

6.3.3. Preparation of emulsions

Continuous phase dispersions of non-texturized WPC (control), TWPC-70, and TWPC-90 were prepared at a fixed concentration of 22% (w/w), by stirring in deionized water for 2 h at room temperature followed by overnight storage at 4 °C. After two hours of mixing, TWPC solutions formed a highly viscous paste resembling a weak cold-set gel, while non-texturized WPC was a shear thinning liquid. Emulsions containing 40%, 60%, and 80% (w/w) soybean oil were prepared by weighing appropriate amounts of protein pastes/solutions and oil in glass containers followed by mixing using a high-speed dispersing unit; IKA Ultra Turrax, T25 basic (IKA Works, Inc., Wilmington, NC, USA)

at 11 000 rpm for 3 min at room temperature. A slightly different method was used to prepare emulsions containing 60% and 80% oil due limitations of the high speed mixer. A pre-emulsion containing 50% oil was prepared as described above, followed by the addition of remaining oil at 10 mL/min while mixing with a Toastmaster beater (Toastmaster Inc., Boonville, MO, USA). Sodium azide (0.02%, w/w) was added to prevent microbial growth.

6.3.4. Storage and heat stability of emulsions

In the storage stability study, emulsions containing 40%, 60%, and 80% (w/w) oil were stored at 4 °C, and the physicochemical properties of emulsions were analyzed at days 1, 15, and 30.

In the heat stability study, 12 g of freshly prepared emulsions containing 40% and 80% (w/w) oil were heated at 70, 80, or 90 °C for 20 min. At the end of the heating period, emulsions were immediately cooled in an ice water bath. Emulsions were stored at room temperature for 24 h before further characterization.

6.3.5. Droplet size distribution

Droplet size distribution of emulsion diluted to 0.5% (w/w) in 1% (w/v) sodium dodecyl sulfate (SDS) was measured with a laser light scattering particle size analyzer (MasterSizer 2000, Malvern Instruments Ltd., Worcestershire, UK). Drops of emulsion solution were introduced to the sample presentation unit containing deionized water at 40

°C. The emulsions were stirred (1200 rpm) continuously to ensure sample homogeneity. Data were collected when obscuration rate of 8% was reached. Droplet size was reported as the volume–weighted mean diameter (d_{43}):

$$d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3 \qquad (1)$$

where n_i is the number of droplets of diameter d_i in the *i*th size class.

6.3.6. Determination of protein load

Freshly prepared emulsions were diluted to 10% (w/w) oil in deionized water and centrifuged at 20,000 g for 20 min at 20 °C. The water phase was removed with a syringe and the cream phase re-dispersed in deionized water and then re-centrifuged. Centrifugation was repeated three times. The cream phase was collected, and protein adsorbed at the interface was analyzed. A 0.2 g of sample was placed in nickel foil lined ceramic boat and covered with 1 g of Leco Com-Aid to assist in combustion of higher fat samples. The sample was analyzed by a nitrogen analyzer, Leco TruMac N instrument (Leco Corporation, St. Joseph, MI, USA). The amount of protein load (Γ) was calculated as:

$$\text{Protein load } (\Gamma)\ (\text{mg/m}^2) = \frac{\text{Protein concentration in cream fraction } (\frac{\text{mg}}{\text{mL}})}{\text{Specific surface area } (\frac{\text{m}^2}{\text{mL}})} \qquad (2)$$

The value of specific surface area was obtained from particle size analysis.

6.3.7. Determination of adsorbed proteins

Proteins adsorbed at droplet surfaces in emulsions containing 40% oil were analyzed by SDS-PAGE both under non-reducing and reducing conditions following the procedure of Laemmli (1970). Cream fractions obtained in section 6.3.6 were dissolved in buffer containing 62.5 mM Tris-HCl, 25% glycerol, 2% SDS and 0.01% bromphenol blue, pH 6.8, at the ratio of 1:2. Buffer containing 5% β-mercaptoethanol (β-ME) was used in reducing conditions. The samples were boiled at 100 ℃ for 5 min. The gel was run on a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples (10 μL in each lane) were run on a 20% separating gel and 4% stacking gel based Mini-PROTEAN Precast gel. The running buffer contained 20% Tris and 5% glycine, pH 6.8. The gel was run for 35 min at a constant voltage of 200 V. Staining was carried out using Bio-Safe Commassie Stain (Bio-Rad Laboratories Inc.) for 1 h and then the gel was de-stained with deionized water. Precision Plus Protein™ Standards from Bio-Rad Laboratories containing recombinant proteins in the molecular weight range of 10 to 250 kDa were used as protein markers.

6.3.8. Confocal laser scanning microscopy (CLSM)

Microstructures of stored and heated emulsions were visualized at days 1, 15, and 30, while heat-treated emulsions were evaluated after 24 h of heat treatment using a confocal laser scanning microscope (Leica TCS-SP2, Leica Microsystem Inc., Bannockburn, IL, USA). Emulsions were stained with a mixture of Nile Red (0.01%, w/w) prepared in polyethyleneglycol, glycerol, and deionized water in a proportion of

50/45/5 and Fast Green FCF (0.001%, w/w) dissolved in deionized water. Nile Red was used to visualize the dispersed oil phase that appeared as bright green globules, whereas Fast Green was used to visualize the water/protein phase that appeared as dark or red background. A 40.0x HCX PL/1.25 oil immersion objective lens with additional 4x zoom was used. The green emission range was 500–580 nm and red emission range was 650–730 nm. Confocal illumination was provided by an Argon laser with excitation at 488 nm and a Helium Neon laser (HeNe) with excitation at 633 nm.

6.3.9. Steady shear rheological analysis of stored emulsions

Steady shear ramps from 1.0 to 100 s^{-1} on stored emulsions were performed using a strain-controlled rheometer (ARES, TA Instruments, New Castle, DE, USA) equipped with a cone and plate geometry (25 mm diameter, 0.051 mm gap and 0.1 radians cone angle). All the measurements were performed at 25 °C. The flow curve was fitted to Power Law model;

$$\eta_a = K\dot{\gamma}^{n-1} \qquad (3)$$

where η_a is apparent viscosity (Pa.s), K is consistency index ($Pa.s^n$), $\dot{\gamma}$ is shear rate (s^{-1}) and n is flow behavior index.

6.3.10. Frequency sweep of heat-treated emulsions

Frequency sweep tests of heat-treated emulsions were performed with a strain-controlled rheometer equipped with a cone and plate geometry (25 mm dia, 0.051 mm

gap, 0.1° angle). Frequency was oscillated from 0.1 to 100 rad/s at 1.0 Pa strain within the linear viscoelastic region. The measurements were performed at 25 °C. Frequency dependence of storage modulus (G') and loss modulus (G") were described with Power Law model:

$$G' = a\omega^{x} \quad (4)$$

$$G'' = b\omega^{y} \quad (5)$$

where ω is the frequency of oscillation (rad/s), exponents x and y represent the slopes of the relationship between modulus and frequency, and coefficients a and b represent the magnitudes of G' and G", respectively, at a given frequency.

6.3.11. Statistical analysis

Statistical analysis was performed using One-Way ANOVA by MINITAB (Version 16) (State College, PA, USA). Difference between means was compared using Tukey's test ($p < 0.05$). All determinations were done in triplicate, except for SDS-PAGE where only duplicate analysis was performed.

6.4. Results and Discussion

6.4.1. Visual observations of emulsions

Figure 6.1 shows freshly prepared non-texturized WPC and TWPC-stabilized emulsions containing 40% to 80% (w/w) oil. Consistency of the emulsions increased with increasing oil concentration. Mixing 40% to 80% (w/w) soybean oil with 60% to

20% of TWPC-70 or TWPC-90 powder dispersions (22%, w/w) in water resulted in the formation of cold-set emulsion gels, attributed to the cold-set gel-like consistency of TWPC in water. On the other hand, non-texturized WPC-stabilized emulsions that contained 40% oil formed liquid emulsions, while emulsions with 60% oil formed very viscous liquids. At 80% oil, a paste-like non-texturized WPC emulsion was obtained.

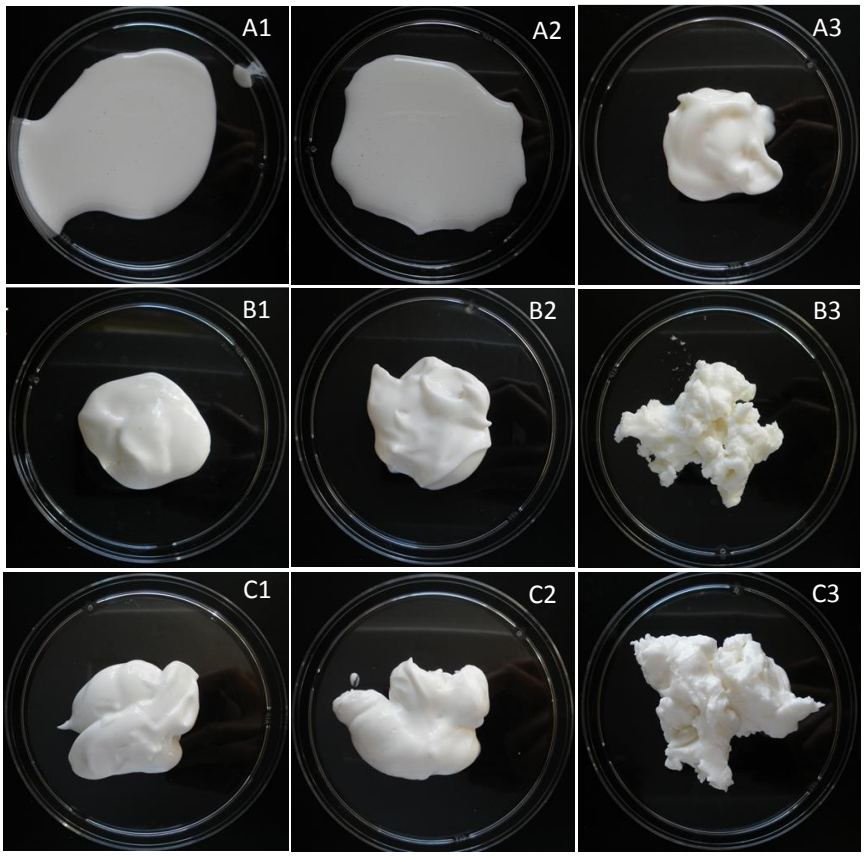

Figure 6.1. Freshly-prepared emulsions stabilized by (A) Non-texturized WPC, (B) TWPC-70, and (C) TWPC-90, containing (1) 40%, (2) 60%, and (3) 80% (w/w) oil.

6.4.2. Storage stability of emulsions

Figure 6.2 shows the droplet size distribution of emulsions containing 40% to 80% (w/w) oil at day 1 of storage at 4 °C. Droplet size increased and size distribution broadened as oil level was increased. This is attributed to insufficient protein to fully coat the droplets. Texturization resulted in significantly ($p < 0.05$) smaller fat droplets and a monomodal droplet size distribution with an almost symmetrical peak in comparison to the larger droplets in non-texturized WPC emulsions. In addition, droplet size distribution of control sample containing 60% oil appeared to be bimodal. Figure 6.3 shows the volume-weighted average droplet size (d_{43}) of fresh and aged emulsions. In all emulsions at any oil levels, the trend in decreasing droplet size followed the order of non-texturized WPC > TWPC-70 > TWPC-90. The droplet sizes of TWPC-90 emulsions ranged from 3.63 to 7.43 μm, whereas control samples formed significantly larger droplets, ranging from 6.45 to 13.73 μm, at 40 to 80% oil. The confocal micrographs of the emulsions are consistent with the particle size data. The increase in droplet size was largely due to flocculation rather than coalescence. In contrast, larger fat droplets with wider size distribution were observed in emulsions made with WPC and β-lg pre-heated at temperatures in the range of 60 to 95 °C for 5–17 min (Kiokias & Bot, 2005; Moon & Mangino, 2004). This was attributed to reduced surface active properties of the excessively aggregated proteins (Li-Chan, 1983; Millqvist-Fureby, Elofsson & Bergenstahl, 2001).

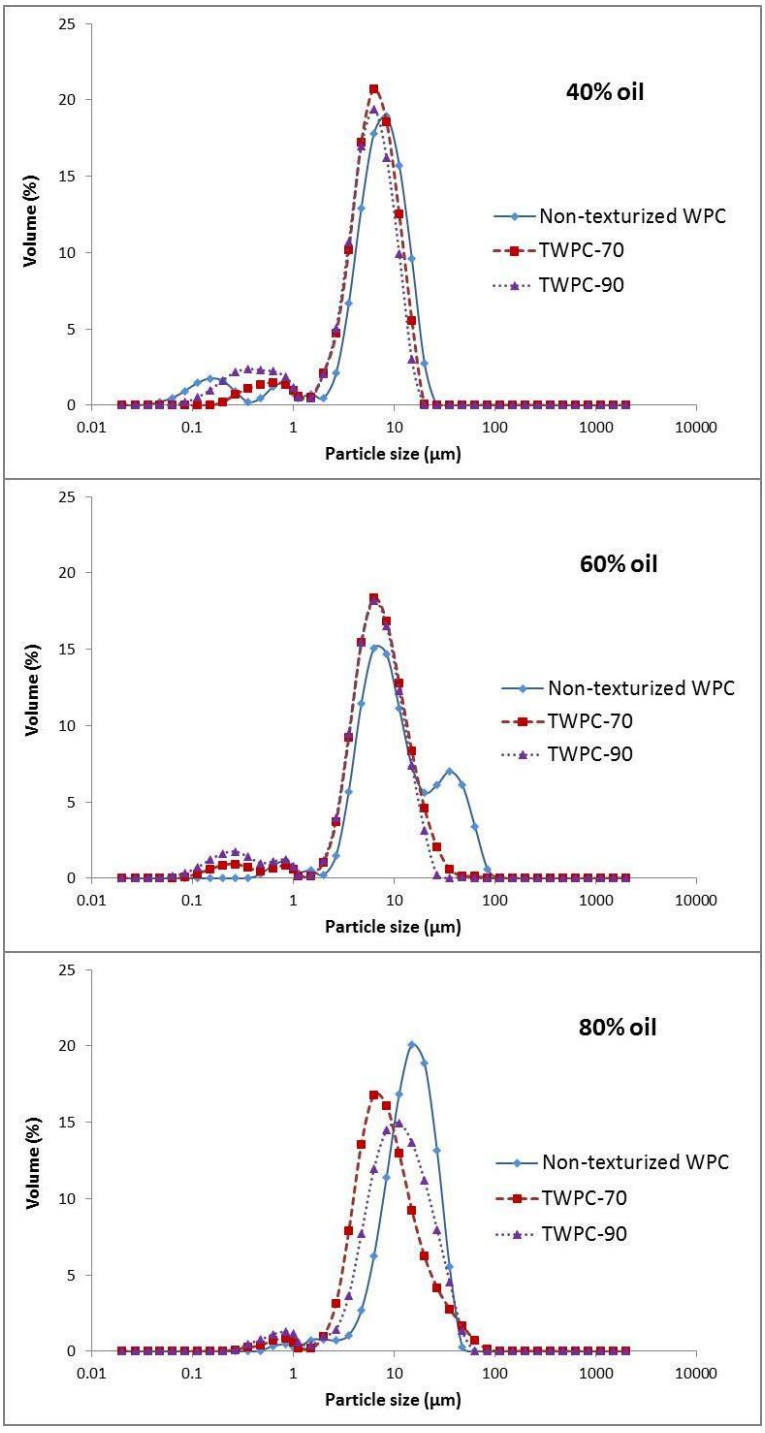

Figure 6.2. Droplet size distributions of emulsions stabilized by non-texturized WPC, TWPC-70 and TWPC-90 stored for 1 day at 4 °C containing 40%, 60%, and 80% (w/w) oil.

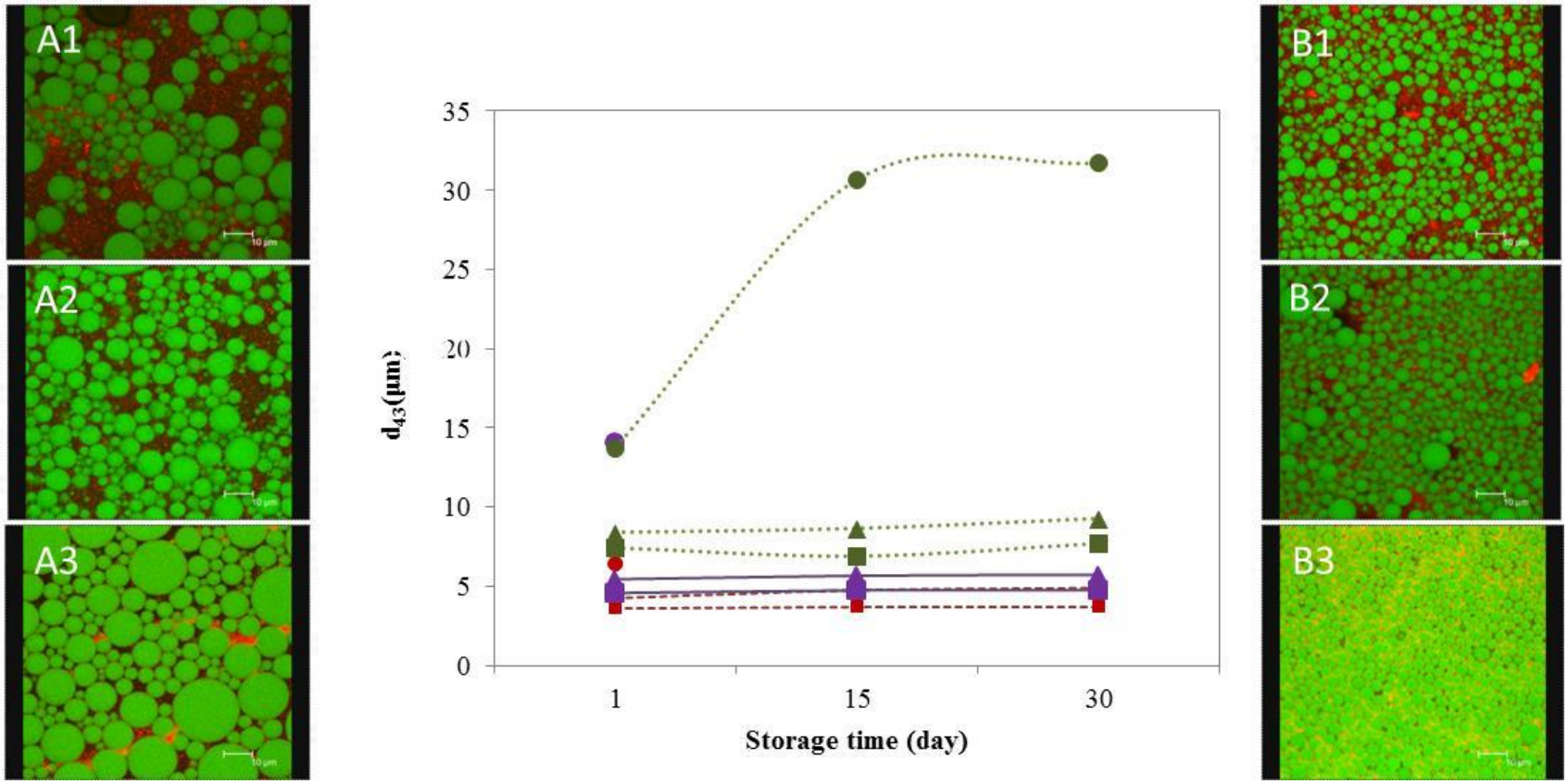

Figure 6.3. Changes in volume-weighted average diameter (d_{43}) of non-texturized WPC (○), TWPC-70 (Δ), and TWPC-90 (□) stabilized emulsions containing 40% (-----), 60% (——), and 80% oil (·····), stored for 30 days at 4 °C. Confocal images represent (A) non-texturized WPC and (B) TWPC-90 stabilized emulsions containing (1) 40%, (2) 60%, and (3) 80% (w/w) oil at day-1. TWPC-70 (not shown) exhibited similar behavior as TWPC-90 emulsions.

The differences in the size of droplets of non-texturized WPC emulsions, compared with TWPCs, might arise from several factors. Previous studies had shown that native proteins formed smaller droplets in comparison with denatured proteins, which was attributed to a higher diffusivity of the former towards the oil–water interface (Fainerman et al., 2006; Millqvist-Fureby et al., 2001). However, in the present study, other factors such as increased surface hydrophobicity (Nor Afizah & Rizvi, 2013) and flexibility of proteins may play a greater role in regulating the emulsifying capacity of TWPC. The greater exposure of aromatic hydrophobic groups due to shear during extrusion increased the affinity of the TWPC towards the interface. The larger droplet size in non-texturized WPC and TWPC-70 compared to TWPC-90 was due to the compact globular structure of native WP and partially denatured protein, respectively. This may have led to fewer exposed hydrophobic groups and fewer contact points at the interface.

Upon storage, non-texturized WPC emulsions that contained 40% and 60% oil underwent phase separation at day 7 and 14, respectively, thus the samples were not further evaluated. The emulsion with 80% oil showed a significant increase ($p < 0.05$) in droplet size at day 15, resulting in an average droplet size of 30.67 μm. As observed in confocal micrograph, the emulsion consisted of some large non-spherical droplets, which implied coalescence. On the other hand, except for TWPC-70 at 40% oil that exhibited a slight increase (11.8–14.9%) in droplet size, all the other TWPC emulsions exhibited excellent droplet stability throughout the storage period (Figure 6.3). The present results were in agreement with Kokias and Bot (2005) who reported that pre-heated and acidified WPC emulsions were relatively stable during storage at 5 °C over a period of 60

days (~30% increase), attributed to the presence of WP aggregates (Dybowska, 2011), while the stability of native β-lg emulsions decreased by more than four times within 6 days of storage at room temperature (Tcholakova, Denkov, Sidzhakova, & Campbell, 2006).

To confirm the role of protein membrane thickness on emulsion stability, protein load (Γ) was determined for freshly prepared emulsions. TWPC emulsions had significantly higher (6.0–23.3 mg/ml) adsorbed proteins, in contrast to the control samples (2.17–6.33 mg/ml) at any oil level (Table 6.1). As expected, the amount of adsorbed protein significantly decreased with increasing oil content. Although TWPC emulsions had greater amounts of adsorbed proteins, the protein loads for most of droplet-coated TWPC emulsions were not significantly different from control samples, ranging from 1.24 to 2.78 mg/m^2. These findings were attributed to the smaller droplets of TWPC emulsion that had larger specific surface areas (1.93 – 6.83 m^2/mg), in comparison to smaller specific areas of non-texturized WPC emulsions (0.81 – 3.23 m^2/mg). Similarly, several authors (Das & Chattoraj, 1980; Tornberg, 1978) have also reported lower protein loads in WPC and BSA stabilized emulsions as the droplets size increased and attributed it to the lower surface area. The present results indicated that the droplets were stabilized by monolayer protein film, in which individual protein molecules are densely packed (Mellema & Isenbart, 2004). On the other hand, the protein loads of TWPC-70 and TWPC-90 emulsions containing 40% and 80% oil, respectively, were significantly higher (3.65 – 4.90 mg/m^2), implying a thicker protein interfacial layer. Based on the published report that a limiting surface load of 3.2 mg/m^2 suggests multilayer protein film (Hunt & Dalgleish, 1994), it is reasonable to suggest that

multilayer protein adsorption took place in emulsions of TWPC-90 with 80% oil and TWPC-70 with 40% oil.

Table 6.1. Specific surface area, amount of adsorbed proteins and protein load in freshly prepared emulsions containing 40 to 80% (w/w) oil[1].

Samples	% oil	Specific surface area (m^2/mL)	Adsorbed protein (mg/mL)	Protein load (mg/m^2)
Non-texturized WPC	40	3.23 ± 0.32^{cd}	6.33 ± 0.58^{de}	1.96 ± 0.18^{b}
	60	1.21 ± 0.06^{cd}	2.50 ± 0.00^{f}	2.07 ± 0.10^{c}
	80	0.81 ± 0.01^{bd}	2.17 ± 0.58^{f}	2.67 ± 0.55^{c}
TWPC-70	40	6.42 ± 0.02^{b}	23.33 ± 2.08^{a}	3.65 ± 0.46^{a}
	60	3.78 ± 0.72^{d}	6.00 ± 0.00^{de}	1.63 ± 0.28^{b}
	80	1.93 ± 0.12^{bc}	5.33 ± 0.29^{e}	2.78 ± 0.25^{c}
TWPC-90	40	6.83 ± 0.46^{cd}	14.00 ± 1.00^{b}	2.06 ± 0.27^{a}
	60	6.44 ± 0.87^{d}	7.83 ± 0.29^{cd}	1.24 ± 0.20^{a}
	80	1.94 ± 0.06^{a}	9.50 ± 0.50^{c}	4.90 ± 0.29^{c}

[1] Means with the same superscript within a column are not significantly different ($p < 0.05$).

These findings also suggest that the stability of emulsion cannot be explained by the protein load data alone. The strength of protein-lipid interactions at the interface also affects the stability of TWPC emulsions. The greater exposure of aromatic hydrophobic residues in TWPC (Nor Afizah & Rizvi, 2013) enhanced the protein–lipid interactions at the interface which made the proteins more difficult to desorb or for droplets to coalesce during storage. It has been reported that coalescence stability of β-lg stabilized emulsions was related to the strength of the protein interfacial layer. β-lg with higher protein surface hydrophobicity was reported to be tightly bound at interface and led to irreversible protein adsorption (Das & Kinsella, 1990). The slight destabilization of TWPC-70 emulsion with 40% oil may perhaps be attributed to the state of adsorbed proteins which consisted of both native and denatured proteins. The former is known to bind loosely at the interface which leads to reversible adsorption (Das & Kinsella, 1990; Dickinson & Hong, 1994).

Comparison of the electrophoretic patterns of adsorbed proteins obtained from freshly prepared emulsions (40% oil) (Figure 6.4A) indicated the presence of monomeric α-la (14.4 kDa) and β-lg (18.3 kDa), dimeric β-lg–α-la (25 to 37 kDa) and some protein aggregates between 160 kDa and 250 kDa. The protein band between 160 and 250 kDa may have consisted of heavy chain immunoglobulin aggregates (MW 160 kDa). All the TWPC emulsions displayed the presence of high molecular weight polymers larger than 250 kDa, indicated by the protein bands that were unable to penetrate the gel. Non-texturized WPC contained less adsorbed α-la than TWPCs, as indicated by lower intensity of the protein band. When the protein fractions were analyzed under reducing conditions, protein bands representing bovine serum albumin (BSA), transferrin, and

lactoferin were observed, protein aggregates at 160 kDa disappeared, and the intensity of high molecular weight polymers (> 250 kDa) of TWPCs was reduced, indicating that the aggregates were cross-linked partly by disulfide bonds. The fact that the dimers were not dissociated by β-ME indicated that they were formed predominantly by non-covalent linkages.

Storage of the emulsions increased the intensity of protein aggregates (Figure 6.4B), particularly in TWPC-70 samples, implying time-dependent protein polymerization via disulfide bonding at the droplet interfaces. The inter-film polymerization of the adsorbed proteins has been shown to result in a decrease in emulsion stability (Damodaran & Anand, 1997; Dickinson & Matsumura, 1991; McClements et al., 1993a; Tcholakova et al., 2006). Therefore, the relatively low storage stability of non-texturized WPC emulsions may be attributed to inter-droplets aggregations via sulfhydryl/disulfide interchange reactions. In TWPC-70 emulsions, both inter- and intra-film protein polymerizations are expected and the later has been proposed to increase the viscoelasticity of protein membrane (Dickinson et al., 1990; Dickinson & Matsumura, 1991), and thus the stability of TWPC emulsions. Significant additional protein polymerization during storage was not observed in TWPC-90, and this was attributed to its already extensively denatured proteins. It is thus reasonable to conclude that the high storage stability of TWPC emulsions was due to their higher surface hydrophobicity that resulted in irreversible protein adsorption and greater strength of interfacial layer due to aggregated proteins at interface that provides greater steric stabilization. The results also implied that TWPC-90, which contains more denatured protein, should indeed serve as a better emulsifier and stabilizer.

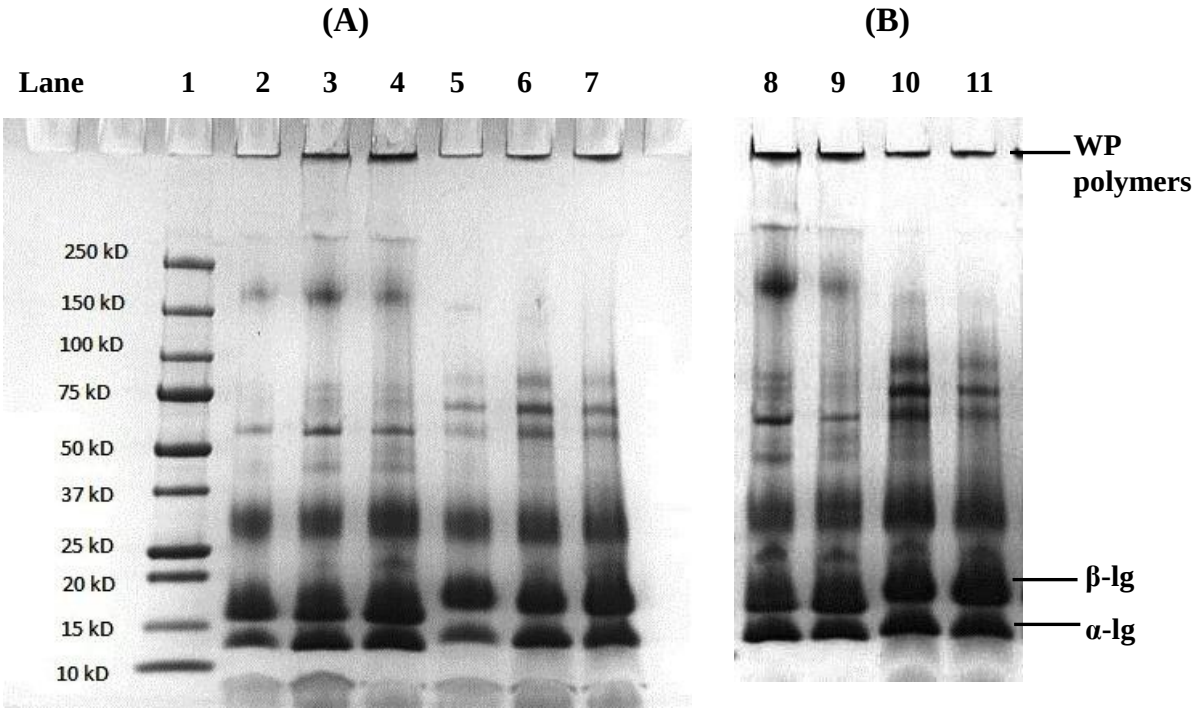

Figure 6.4. SDS-PAGE of adsorbed proteins from non-texturized WPC, TWPC-70 and TWPC-90 stabilized emulsions (40% oil) under non-reducing and reducing conditions. The cream fractions obtained from (A) freshly prepared emulsions, and (B) emulsions stored for 15 days at 4 °C. Lane 1 is protein markers; Lanes 2-4 and 8-9 are samples under non-reducing conditions; Lanes 5-7 and 10-11 are samples under reducing conditions. Adsorbed proteins at Lanes 2 and 5, represent non-texturized WPC; Lanes 3, 6, 8 and 10 represent TWPC-70, and; Lanes 4, 7, 9, 11 represent TWPC-90.

6.4.3. Rheological properties of stored emulsions

Figure 6.5A shows the viscosity-shear rate profiles of whey protein emulsions measured at day 1 of storage at 4 °C. Apparent viscosity of all samples decreased with increasing shear rate, implying a shear-thinning behavior, attributed to droplet deflocculation (McClements, 1999). As the oil phase weight fraction increased, flow curves expectedly moved to higher viscosities. This can be attributed to an increase in the packing of oil droplets in the system, making the system more compact. The droplets acted not only as active fillers but also as anchor points, when they are fitted into the protein gel matrix (Chen & Dickinson, 1998; Sok Line et al., 2005; van Vliet, 1988). The significantly high apparent viscosity observed in emulsions containing 80% oil despite their low protein content may be attributed to the greater packing of oil droplets. The shear-thinning character of non-texturized WPC emulsion that contained 40% oil was less noticeable, as indicated by a high flow behavior index (n = 0.88). All TWPC emulsions exhibited greater pseudoplastic properties at any oil level, compared with the control samples, as indicated by lower flow behavior index (n = 0.01 to 0.17). The pseudoplastic character of the emulsions following 30 days of storage remained constant (Figure 6.5B).

Figure 6.6 shows changes in the consistency index of the emulsions measured at 15-day intervals for 30 days. The TWPC emulsion gels exhibited greater consistency indices than gels made with WPC, with a 14.1 to 1075.0-fold greater consistency index at all oil levels tested. TWPC-90 emulsions exhibited the highest consistency indices, implying stronger cold-set emulsion gels. This was attributed to the extensively denatured protein in TWPC-90 that formed a highly viscous continuous phase. Similarly,

more viscous or firmer emulsions were obtained with WPC that was pre-heated at higher temperatures (50–90 °C), due to increasing levels of denatured proteins (Dybowska, 2011; Kiokias & Bot, 2006). All the TWPC emulsions were stable while the non-texturized WPC emulsions at 40 and 60% oil levels were phase separated during storage. The TWPC-70 emulsions at similar oil levels exhibited a slight reduction in consistency indices at day 15, and further storage to 30 days did not significantly change the consistencies. In contrast, TWPC-90 emulsions exhibited insignificant changes throughout the storage period. In emulsion containing 80% oil, a continuous reduction in consistency index was observed in non-texturized WPC, leading to a 30% decrease at day 30 of storage. TWPC-70 showed a slight reduction (7.8%), while TWPC-90 emulsions exhibited a slight increase (11.6%) after 30 days of storage. In contrast, a much higher increase of 50% to 100% in the firmness was observed in pre-heated, acidified WPC emulsion gels stored at 5 °C over a period of 60 days (Kiokias & Bot, 2005), indicating less stable emulsions in comparison to TWPC emulsions.

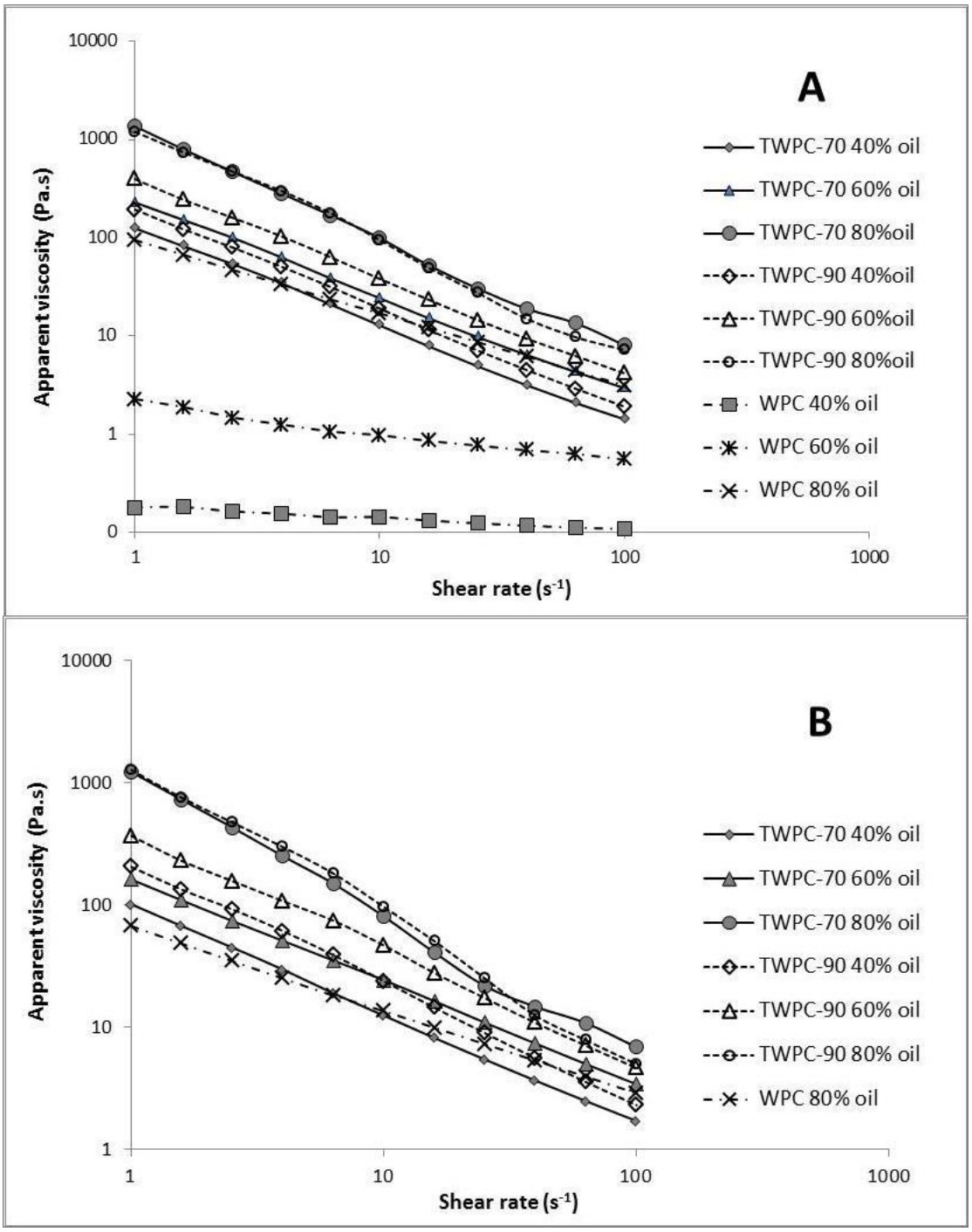

Figure 6.5. Changes in apparent viscosity of WPC and TWPC stabilized emulsions containing 40%, 60% and 80% (w/w) oil at (A) day 1, and (B) day 30 of storage at 4 °C.

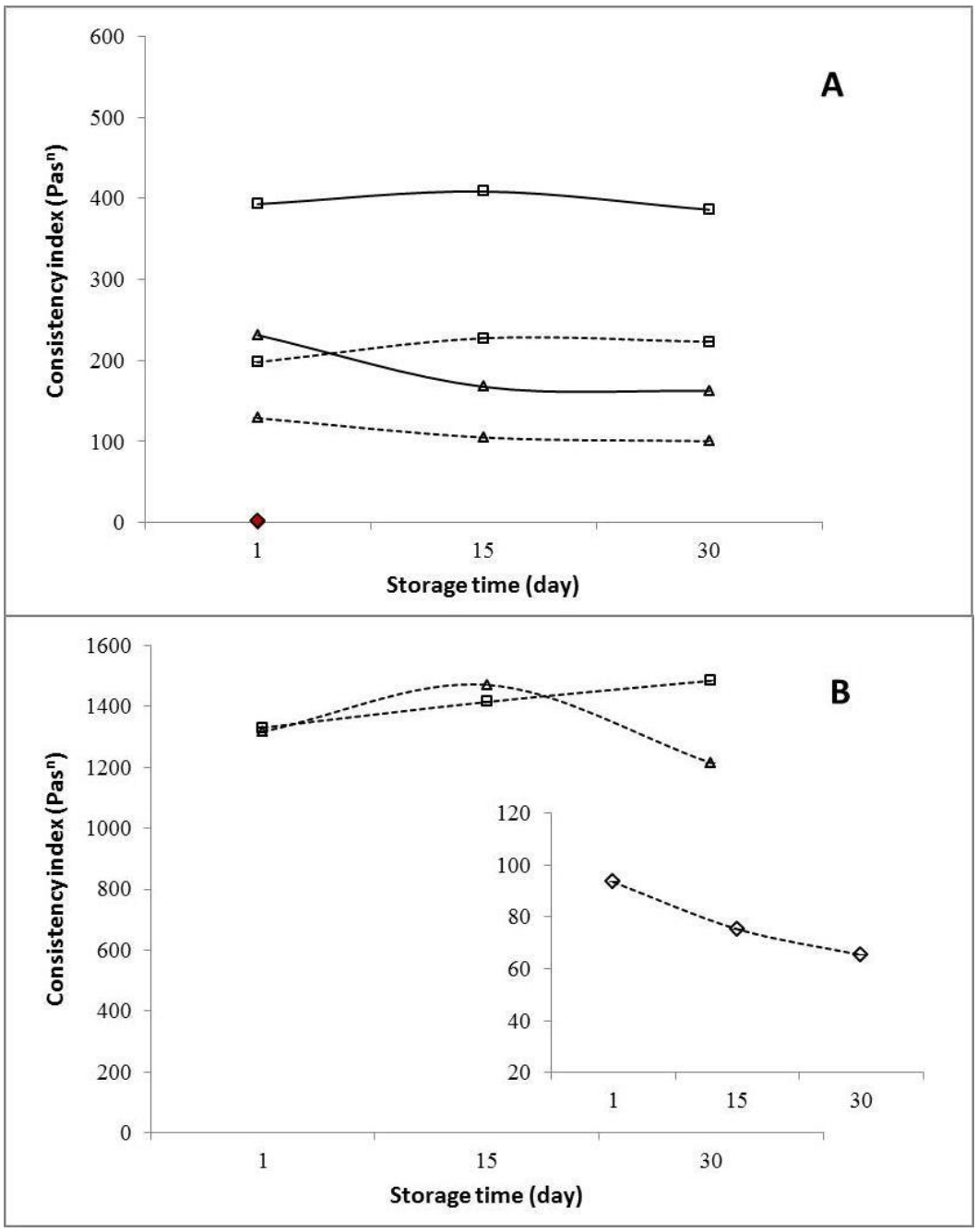

Figure 6.6. Changes in consistency index of non-texturized WPC (◊), TWPC-70 (Δ), and TWPC-90 (□) stabilized emulsions over storage for 30 days at 4 °C containing (A) 40% (-----) and 60% (——) oil, and (B) 80% oil. The small insert in (B) represents non-texturized WPC emulsions.

6.4.4. Thermal stability of emulsions

Changes in volume-weighted mean droplet size (d_{43}) of heated whey protein emulsions are shown in Figure 6.7. The size of droplets in the non-texturized WPC emulsions at 40% oil increased after heating and consequently the distribution of droplet sizes broadened (Figure 6.8). An abrupt increase in droplet size from 8.0 μm (non-heated) to 15.4–15.6 μm was observed in non-texturized WPC emulsions when heated at 70 and 80 °C, while at 90 °C droplet size change was not significant. Droplet aggregation of WPI- and WPC-stabilized emulsions has been shown to occur in the range of 65–80 °C as temperature was raised above 80 °C (Monahan, McClements, & Kinsella, 1993; Demetriades et al., 1997; Sliwinski et al., 2003). In contrast, all TWPC emulsions exhibited better stability as indicated by smaller increases in d_{43} when heated at 70 °C. At 90 °C, the droplet size of TWPC-90 emulsion increased from 4.0 μm (non-heated) to 5.6 μm, whereas TWPC-70 increased from 6.1 μm (non-heated) to 9.7 μm. Emulsions that contained 80% oil performed differently. Non-texturized WPC emulsions were stable at 70 °C, but the droplet size increased when heated to 80 or 90 °C. The greatest destabilization was observed at 80 °C, as indicated by high increase (42.1%) in d_{43}, along with the broadening of the droplet size distribution (Figure 6.9). The d_{43} of TWPC emulsions at similar oil level remained almost constant after heating at all temperatures. The droplet size distribution of the TWPC-70 emulsion showed a slight broadening, whereas the distribution in heated TWPC-90 emulsions remained constant even at high temperature, implying excellent thermal stability. The results also correlate well with confocal microscopy observations. The droplets in non-texturized WPC coalesced to form large, non-spherical droplets at 80 °C (Figure 6.7). In contrast, no noticeable

change was observed in the droplets of TWPC emulsions when heated at any temperature.

The extent of protein denaturation at the oil-water interface and in the continuous phase influences the thermal behavior of emulsions. The increase in droplet size of non-texturized WPC emulsions when heated at or below 80 °C was attributed to the increase in surface hydrophobicity of fat droplets due to partial denaturation of native protein adsorbed at interface (Demetriades et al., 1997; Monahan et al., 1996). Similar behavior was expected in TWPC-70 but at higher temperature (90 °C), due to greater thermal stability of the acidic texturized protein. The greater destabilization of emulsions containing 40% than those with 80% oil was possibly due to the presence of greater amount of non-adsorbed protein in the former, which acted as 'glue' to hold the aggregated droplets together (Euston et al., 2000).

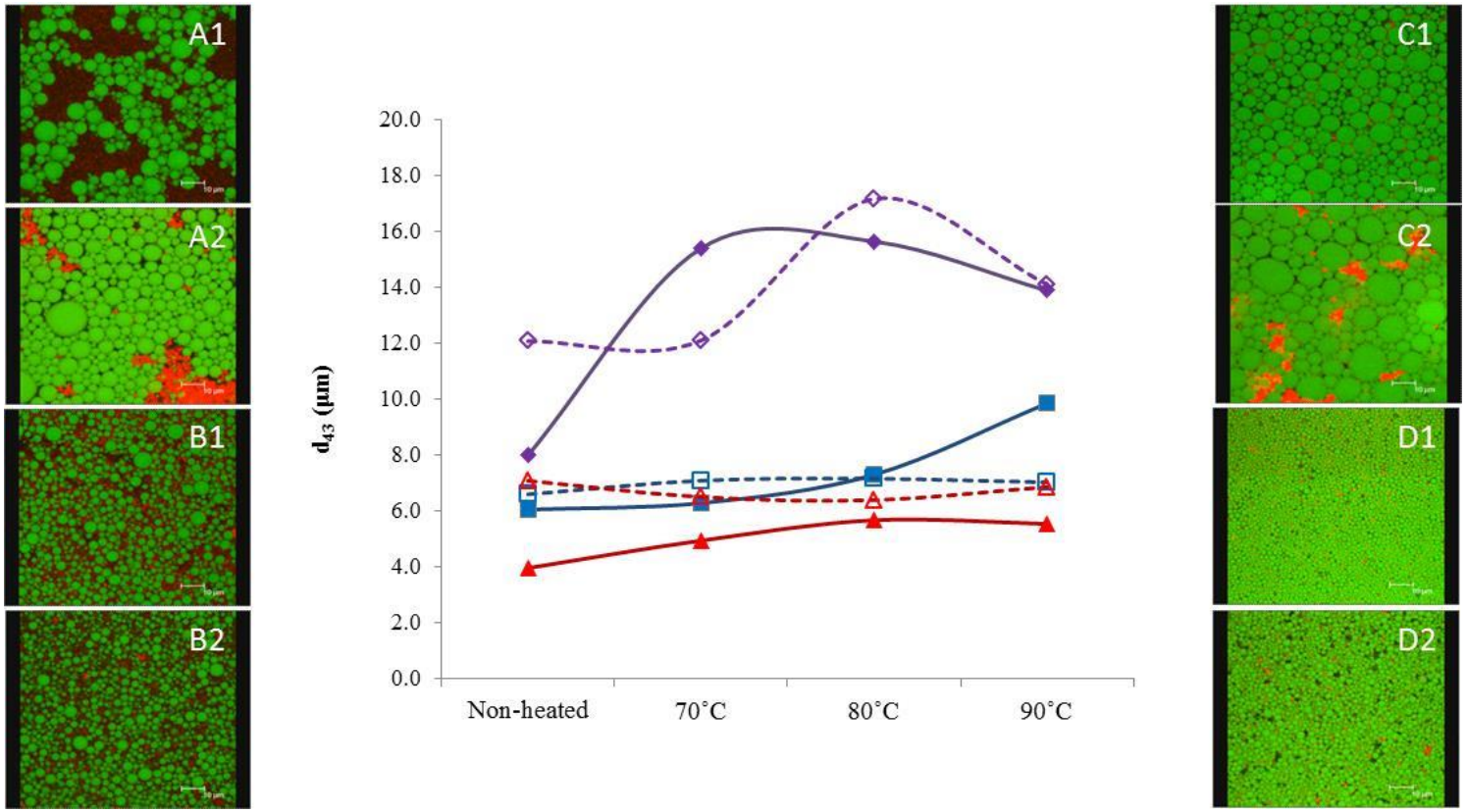

Figure 6.7. Changes in volume-weighted average diameter (d_{43}) of non-texturized WPC (◊), TWPC-70 (□), and TWPC-90 (Δ) containing 40% (filled symbols, ——) and 80% oil (empty symbols, -----) heated at 70-90 °C for 20 min. CLSM images represent non-texturized WPC (A and C) and TWPC-90 (B and D) emulsions containing 40% (A and B) and 80% oil (C and D). The emulsions were (1) non-heated and (2) heated at 80 °C. The TWPC-70 stabilized emulsions exhibited similar behavior as TWPC-90 stabilized emulsions.

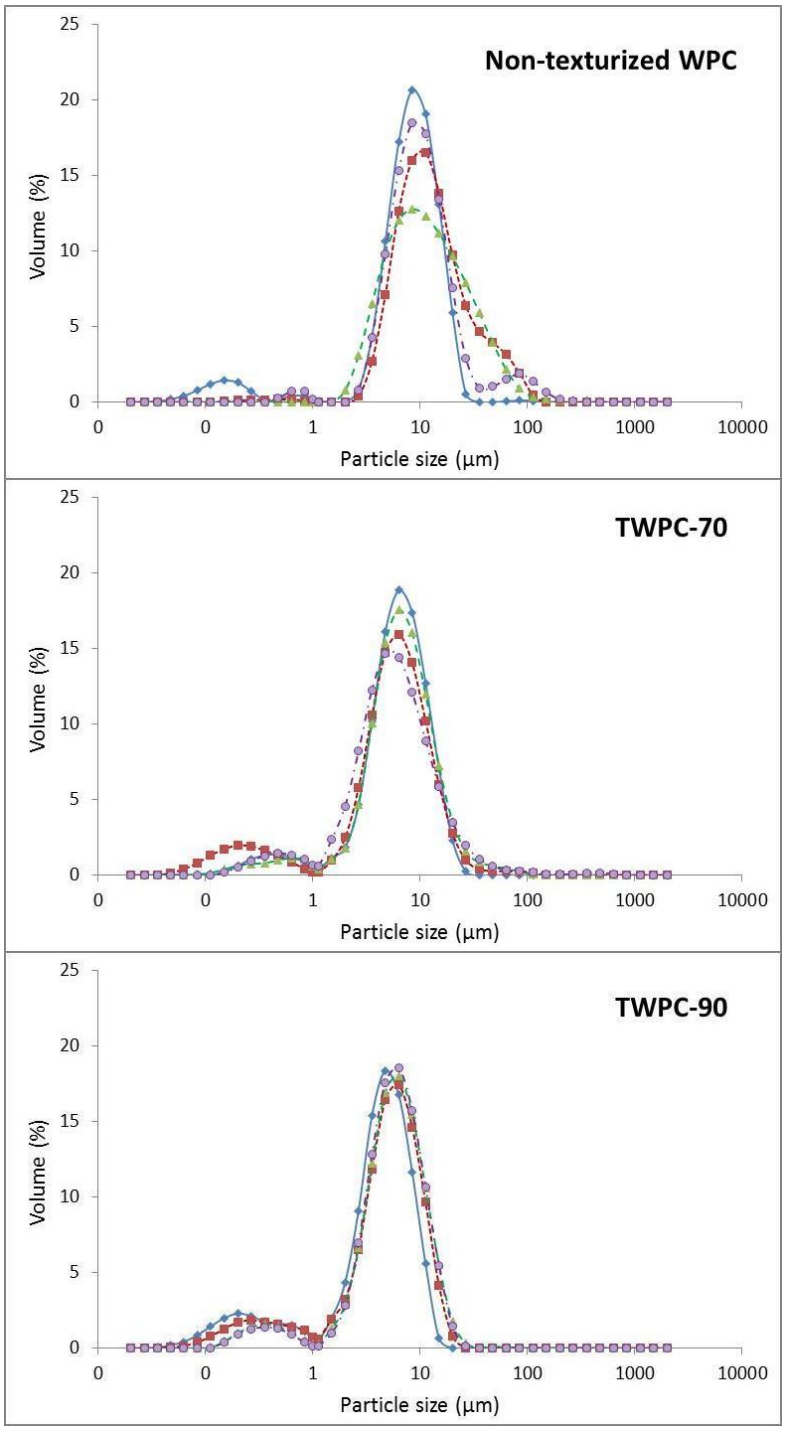

Figure 6.8. Droplet size distributions of emulsions containing 40% (w/w) oil stabilized by non-texturized WPC, TWPC-70, and TWPC-90. The emulsions were non-heated (◊), heated at 70 °C (□), 80 °C (Δ), or 90 °C (○) for 20 min.

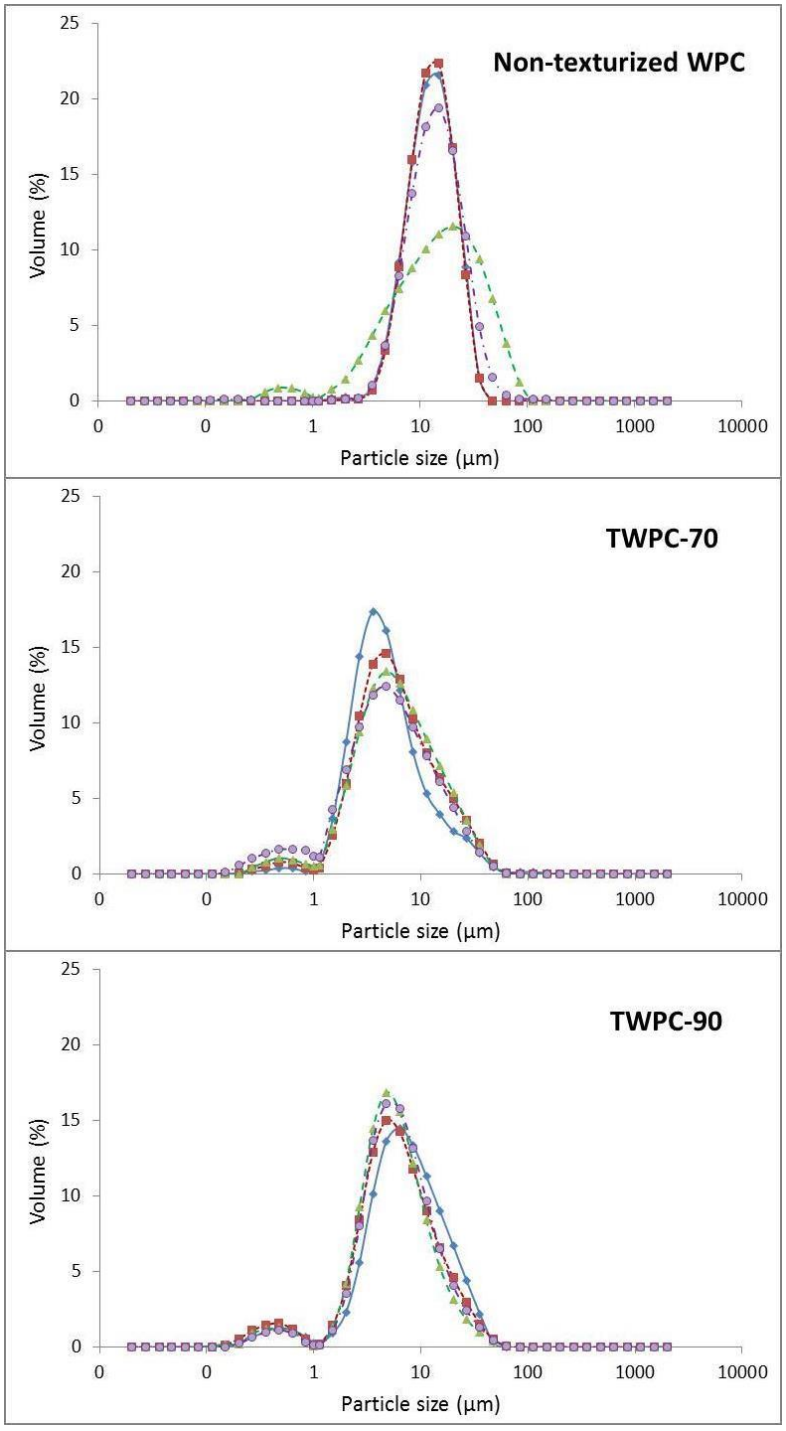

Figure 6.9. Droplet size distributions of emulsions containing 80% (w/w) oil stabilized by non-texturized WPC, TWPC-70, and TWPC-90. The emulsions were non-heated (◊), heated at 70 °C (□), 80 °C (Δ), or 90 °C (○) for 20 min.

The changes in conformation and composition of adsorbed protein due to heat treatment were determined by analyzing the electrophoretic pattern of the cream fractions of the emulsions. In non-texturized WPC emulsions analyzed under non-reducing conditions, the intensity of adsorbed monomeric β-la and α-la bands gradually decreased with increasing temperature, and the α-la band disappeared when heating was done at 90 °C (Figure 6.10). Several authors have demonstrated that α-la was displaced by the β-lg from the aqueous phase during heating of emulsions at 90-121 °C (Ye, 2010; Singh & Ye, 2009). However, the increase in intensity of β-lg band was not observed, therefore, it can be proposed that α-la was not bound at the interface as tightly as the β-lg molecules. Under reducing conditions, slight increases in the intensities of monomeric and dimeric protein bands were observed and a faint band of α-la reappeared at 90 °C, indicating proteins polymerization occurred via disulfide bonds. However, high molecular weight polymers were not observed in any heated non-texturized WPC emulsions, possibly attributed to the inter-film protein polymerization that resulted in weaker interactions between adsorbed protein and fat surface, causing the proteins to easily desorb during heating.

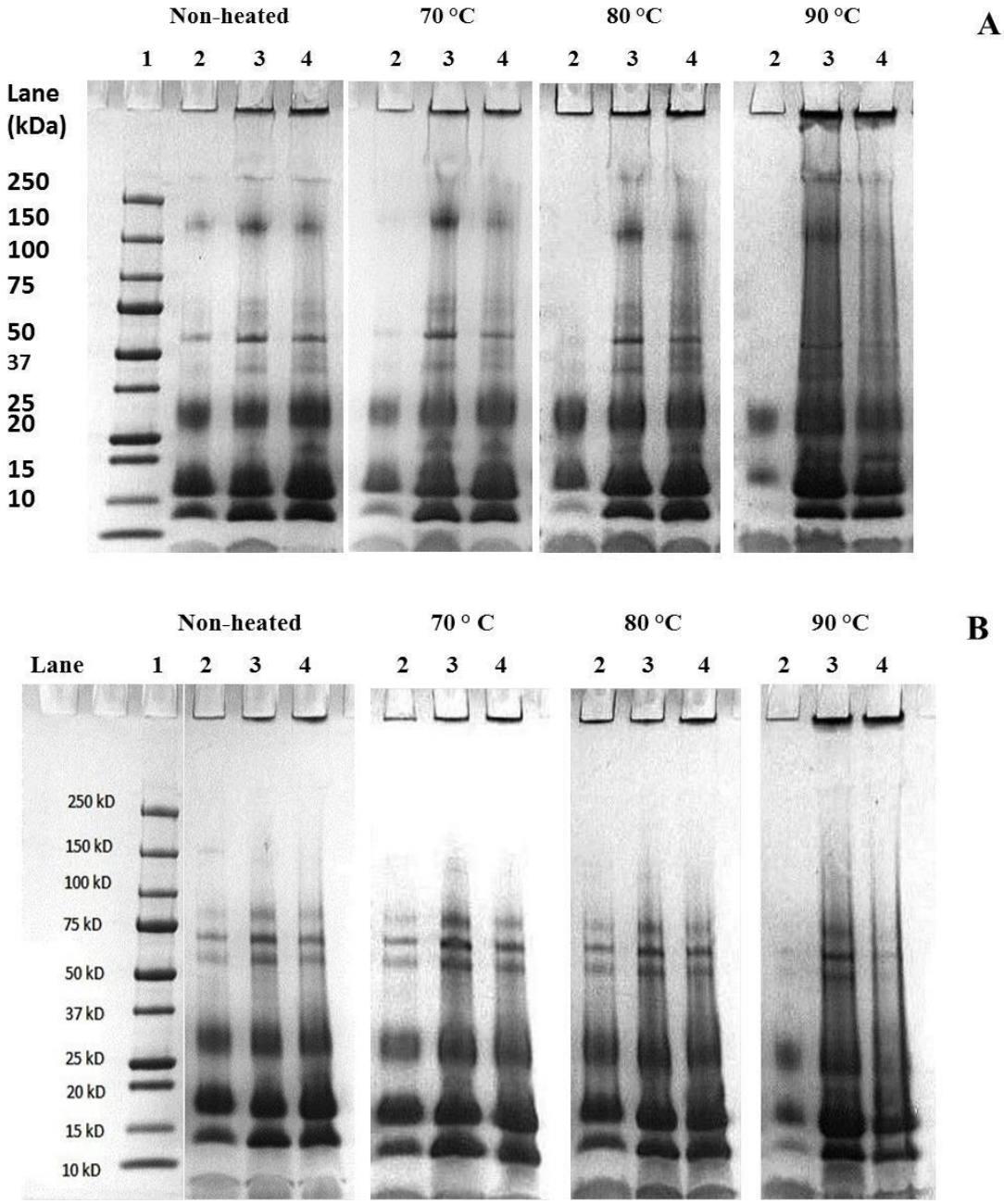

Figure 6.10. SDS-PAGE of the cream fractions of emulsions (40% oil) heat treated at 70-90 °C for 20 min under (A) non-reducing, and (B) reducing conditions. Lane 1 is protein markers. Lanes 2, 3, and 4 corresponds to non-texturized WPC, TWPC-70, and TWPC-90, respectively.

In contrast, heating the TWPC emulsions at ≥ 80 °C showed a gradual increase in the amount of high molecular weight polymers with increasing temperature, most prominent at 90 °C, as indicated by increased intensity of protein band that cannot penetrate the gels. Streaking of the band, which indicated extensive protein polymerization was observed at 90 °C in both TWPC emulsions, particularly in TWPC-70. Under reducing condition, the intensities of the protein aggregates and streaking of the bands decreased, implying that the polymerization occurred partially via disulfide bonds. The aggregation of protein at the interface perhaps not only increased the thickness, but also the rigidity of the protein membrane due to inter-film polymerization. Therefore, it can be concluded that the degree of denaturation in protein used to make the emulsion plays an important role in thermal stability of emulsion because it influences the conformation and composition of protein at the interfacial layer.

6.4.5. Dynamic rheological properties of heated emulsions

The mechanical spectra of heated emulsions are shown in Figure 6.11 and the Power Law parameters for the frequency dependence of G' are listed in Table 6.2. At 40% oil, non-heated, control WPC emulsion shows higher G" than G' throughout the frequency tested, implying a liquid-like behavior. The moduli showed a strong dependence on frequency with an exponent *x* of 0.661. When heated to 70 °C, G' overlapped with G", indicating the beginning of gel formation. Heating the emulsions at 80 or 90 °C resulted in G' > G" that were fairly independent of frequency, as indicated by the exponent *x* of 0.077–0.085. The elasticity of the gels at higher temperatures was

greatly increased (~6000-fold), as indicated by a change in parameter *a* from an initial value of 0.39 Pa to 1934 - 2386 Pa

On the other hand, G' of all TWPC emulsions at 40% oil was greater than G", suggesting a dominant solid-like elastic behavior. TWPC-70 emulsions submitted to thermal treatment at ≥ 80 °C exhibited a significant increase in G' by 5.0- to 6.3-fold, as indicated by enhanced parameter *a* (8230–10434 Pa) from an initial value of 1650 Pa. When heated at 80 °C, the TWPC-70 emulsion gel had a G' value comparable to that of TWPC-90. When heated at 90 °C, the value of G' of TWPC-70 surpassed that of TWPC-90, as indicated by the high parameter *a* value of the former. The G' of TWPC-90 emulsion remained almost constant even when subjected to high heat treatment as shown by the values of parameter *a* (5795–7727 Pa) and exponent *x* (0.164–0.208).

In all samples at 80% oil, G' was higher than G" and the moduli were independent of frequency as indicated by a low exponent *x* ranging from 0.036–0.131, implying a relatively strong solid-like behavior. An exponent *x* near zero indicates a characteristic for a fully cured gel (Resch & Daubert, 20024). Following heating at 70 °C, there was a slight reduction (0.8- to 0.9-fold) in the values of G' for the non-texturized WPC and TWPC-70 emulsion gels. This may be attributed to disruption of inter-droplet attractive interactions following rearrangement of conformational structure of adsorbed proteins (Dickinson & Parkinson, 2004). Heating both emulsions at 80–90 °C caused only a minimal increase in G' (1.1–1.3-fold). Stable G' was observed in TWPC-90 emulsions at all heating temperatures. At 90 °C both TWPC-70 and TWPC-90 emulsion gels showed comparable parameter *a* values, but a lower exponent *x* (0.102) was obtained for TWPC-90, suggesting that the later had greater elastic properties. The

increase in G' during heating of non-texturized WPC and TWPC-70 emulsions was attributed to the formation of protein networks following denaturation and aggregation of native proteins during heating. The excellent behavior of TWPC-90 emulsions upon heating even at 90 °C can be attributed to the already extensively denatured proteins during extrusion that was incapable of undergoing additional protein cross-linking.

As shown in Table 6.2, the tan δ of non-texturized emulsions heated at 80° and 90 °C were lower (0.050–0.117) than those of TWPC emulsions (0.100–0.222). The tan δ of emulsions with 80% oil was also lower than for 40% oil emulsion. The lower tan δ suggested that the samples were more elastic. The differing rheological characteristics of non-texturized WPC and TWPC emulsion gels is assumed to be due to differences in the types of major interactions involved in the formation of the gels. In heated, non-texturized WPC samples, the gels were predominantly made up of disulfide bonds that bridged the protein networks and emulsion droplets, while in TWPC emulsions, the gels were predominantly formed by non-covalent interactions at additional sites exposed by extrusion.

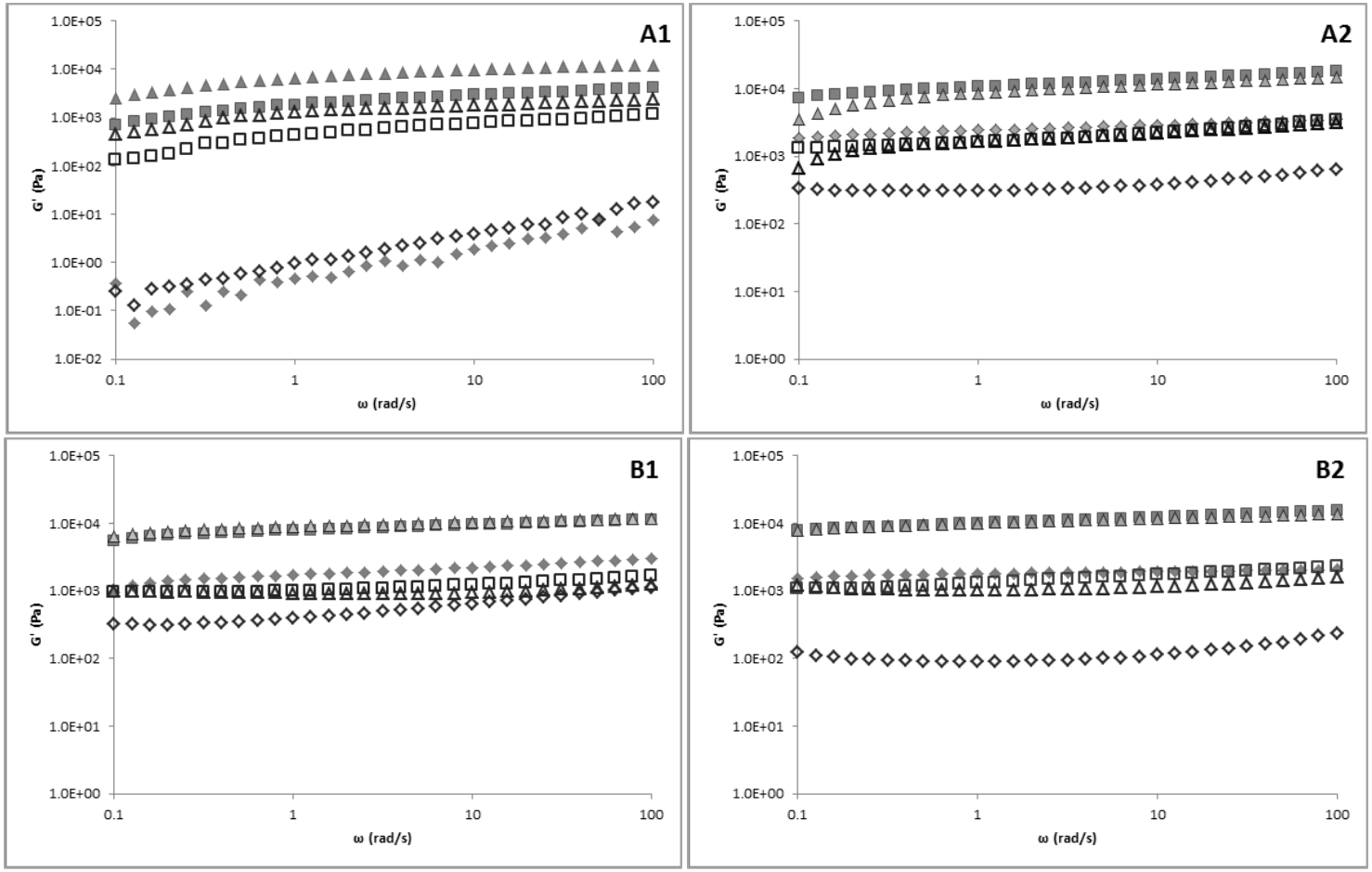

Figure 6.11. Frequency sweep dependence of storage modulus (G', filled symbols) and loss modulus (G", empty symbols) of non-texturized WPC (◊), TWPC-70 (□) and TWPC-90 (Δ) stabilized emulsion containing (A) 40%, and (B) 80% (w/w) oil. The emulsions were (1) non-heated, and (2) heated at 90 °C.

Table 6.2. Power Law parameters of frequency dependence of G' of non-heated and heated emulsions[1].

Treatments	*a*			*x*			Tan δ		
	WPC	TWPC-70	TWPC-90	WPC	TWPC-70	TWPC-90	WPC	TWPC-70	TWPC-90
40% oil									
Non-heated	0.39^{Cc}	1650^{Bd}	5796^{Aa}	0.661^{Aa}	0.233^{Ba}	0.206^{Ba}	3.039^{Aa}	0.225^{Ba}	0.191^{Bb}
70 °C	2.71^{Cc}	2749^{Bc}	6602^{Aa}	0.451^{Ab}	0.166^{Bb}	0.192^{Ab}	0.816^{Aab}	0.188^{Bb}	0.230^{Ba}
80 °C	1634^{Bb}	8230^{Ab}	6987^{Aa}	0.077^{Cc}	0.134^{Bc}	0.208^{Aa}	0.117^{Cb}	0.166^{Bbc}	0.222^{Aa}
90 °C	2386^{Ca}	10434^{Aa}	7727^{Ba}	0.085^{Cc}	0.121^{Bd}	0.164^{Ab}	0.127^{Cb}	0.149^{Bbc}	0.189^{Ab}
80% oil									
Non-heated	1663^{Cab}	7655^{Bbc}	8653^{Ab}	0.131^{Aa}	0.089^{Bc}	0.075^{Ca}	0.226^{Aa}	0.124^{Bb}	0.104^{Ba}
70 °C	1505^{Cb}	6337^{Bc}	9230^{Aab}	0.082^{Ab}	0.107^{Ba}	0.073^{Ba}	0.134^{Ab}	0.148^{Aa}	0.098^{Ba}
80 °C	1730^{Ba}	8537^{Aab}	9410^{Aa}	0.039^{Cc}	0.101^{Ab}	0.074^{Ba}	0.055^{Cc}	0.141^{Aa}	0.100^{Ba}
90 °C	1771^{Ba}	9964^{Aa}	9895^{Aa}	0.036^{Cc}	0.093^{Ac}	0.075^{Ba}	0.050^{Cc}	0.128^{Ab}	0.102^{Ba}

[1]Means with the same lower case superscript within a column at each oil level are not significantly different ($p < 0.05$). Means with the same upper case superscript within a row at each oil level are not significantly different ($p < 0.05$).

6.5. Conclusions

TWPCs produced by the RSCFX process were able to form cold-set emulsion gels that could contain high levels of oil (40–80%). Non-texturized WPC emulsions containing 40–60% oil, on the other hand, could only form gels when heated to a minimum temperature of 80 °C. TWPC produced at high extrusion temperatures (TWPC-90) formed emulsions with higher consistency, smaller fat droplets, and better stability during long-term storage and when heated to 90 °C. The excellent performance of TWPC-90 was due to its extensively denatured proteins that were unable to further polymerize during storage, thus diminishing the probability of inter-droplet interactions and coalescence. The polymerization of adsorbed TWPC-90 during heating enhanced the rigidity of the interfacial layer. The formation of additional protein networks in TWPC-70 emulsions due to heating enhanced their elastic character, comparable in gel strength to TWPC-90. The present results demonstrated that TWPC outperformed WPC as an emulsifier and stabilizer and that TWPC-90 offers the best properties in this regard.

6.6. References

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