# PHYSICO-CHEMICAL PROPERTIES OF WHEY PROTEIN CONCENTRATE TEXTURIZED BY REACTIVE SUPERCRITICAL FLUID EXTRUSION

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# PHYSICO-CHEMICAL PROPERTIES OF WHEY PROTEIN CONCENTRATE TEXTURIZED BY REACTIVE SUPERCRITICAL FLUID EXTRUSION

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Whey proteins (WP) are widely used in a variety of food formulations and constitute a significant share of the dairy ingredients market. In this research WP functionalities were modified using a novel reactive supercritical fluid extrusion (SCFX) process. High pressure extrusion of WP under different pH conditions and in the presence of mineral salts, combined with a delicate control of heat, shear, and internal environments created by introduction of supercritical carbon dioxide (SC-$CO_2$), was used to texturize and develop unique functional properties in commercially available whey protein concentrate (WPC).

A feed formulation comprising (w/w) 94% WPC-80, 6% pre-gelatinized corn starch, 0.6% (WP-starch basis) NaCl, and 0.6% (WP-starch basis) $CaCl_2$ was texturized in a high-pressure extruder at 90 °C and 60% (dry feed basis) moisture in the pH range of 2.89 to 8.16 with 1% (dry feed basis) SC-$CO_2$ injected as a blowing agent. The average specific mechanical energy (SME) input for the process was 57 Wh/ kg. The resulting texturized WPC (tWPC) extrudates were dried, ground into powder, reconstituted in deionized water and evaluated for their rheological and physicochemical properties.

The rheological behavior of tWPC was found to be strongly dependent on the pH and SC-$CO_2$ levels used during extrusion. The highest apparent viscosity ($\eta$=2.06 Pa·s) and elastic modulus ($G'$=10 kPa) values were observed in the tWPC produced at

extremely acidic condition (pH 2.89) with SC-$CO_2$ injection and were significantly higher than those exhibited by the unextruded control ($\eta$=0.008 Pa·s, and $G'$= 0.04 Pa). A 20% (w/w) tWPC dispersion exhibited a highly viscous and creamy texture with particle size in the micron-range (mean diameter ~ 5 µm) which could serve as a thickening/gelling agent or as a fat substitute in food formulations over a wide range of temperatures. The soluble protein content and free sulfhydryl groups of the tWPC decreased by approximately 20% and 16% relative to the unextruded control. The tWPC was completely soluble in the presence of urea (8 M) and sodium dodecyl sulfate (0.5%) without a reducing agent, indicating that the non-covalent interactions (hydrophobic interactions and hydrogen bonds) were mainly responsible for the structural formation of the tWPC.

A homogeneous gel-like emulsion of creamy consistency was also successfully produced by incorporation of corn oil with tWPC dispersion in water serving as the continuous aqueous phase. Only 4% (w/w) tWPC was needed to emulsify 80% corn oil and it showed a higher thermal stability upon heating to 85 ºC. It also showed excellent emulsifying properties (emulsion activity index, EAI, = 431 $m^{-2}$ $g^{-1}$, emulsion stability index, ESI, = 13,500 h) compared to the commercial WPC-80 (EAI = 112 $m^{-2}$ $g^{-1}$, ESI = 32 h). Emulsions prepared with such small amounts of tWPC showed an enhanced adsorption of proteins at the oil-water interface which prevented flocculation and coalescence of the oil droplets, and an increase in the viscosity of the continuous phase which prevented creaming by trapping the oil droplets within the gel matrix. These attributes helped generate very stable oil-in-water emulsions of important utility in food formulations and should be useful in new product development.

# BIOGRAPHICAL SKETCH

The author was born in Kampangphet, Thailand to Mr. and Mrs. Manoi. She received her Bachelor of Science in Agro-Industry from Naresuan University, Thailand in 1996. She joined the Agro-Industry Department of Naresuan University as the Academic Staff, right after graduation. In 1999, she received a Royal Thai Government Scholarship to pursue her Master of Science in Food Process Engineering at Asian Institute of Technology, Thailand and graduated in 2000. In January 2003, she received the Ph.D fellowship from Naresuan University and then joined Prof. Syed Rizvi's group in the field of Food Science at Cornell University.

*To my dad, mom, and sisters, for your love and support*

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# CHAPTER ONE

# INTRODUCTION

## 1.1. Whey proteins

When casein is removed from skim milk, the remaining aqueous phase is designated as whey or milk serum. A further refinement in terminology reflects the method by which the casein is removed from skim milk (Brunner, 1977). If participated with acid at pH 4.6 and 20ºC, the aqueous phase is “acid whey”. If removed by the action of rennin or rennin-like enzymes, as in the production of Cheddar cheese, the aqueous phase is “sweet whey”. Because whey contains 93% water and only 0.6% protein, it is concentrated to produce the various types of whey protein products, including whey protein concentrates (WPC) and whey protein isolates (WPI). WPC include a wide range of products with protein contents from 25-89%. However, the most readily available forms for commercial uses have protein contents of 34% and 80%. WPI are a purer protein than WPC, containing greater than 90% protein, and often utilize ion exchange in their production. Producing WPC and WPI requires that large amount of non-protein compounds be removed, therefore separation technologies have been developed. Manufacturing processes and conditions for whey proteins can vary from producer to producer. Nowadays, the manufacturing technology for whey proteins can be classified into two major categories: membrane technology, which includes cross-flow microfiltration (MF) and ultrafiltration (UF), and ion-exchange chromatography (IE) (Fox, 2003).

The typical production process for WPC includes UF-diafiltration, (optional step: evaporation) and spray drying. Microfiltration is also used to reduce the fat content for the higher protein WPC products. Two different approaches are used to produce WPI, 1) MF, UF-diafiltration, and spray drying and 2) IE coupled with

concentration (evaporation or UF) and spray drying. Sweet whey, after separation from cheese curd, is often clarified to remove any remaining fines and pasteurized, and most of the residual fat is removed by centrifugal separation (as whey cream). In the MF/UF process used for WPI production, virtually all the fat remaining in the whey is removed during the MF step because it is effectively retained by the membrane. It should be noted that all the soluble whey proteins are concentrated in the MF/UF process, whereas in the IE process some proteins, e.g., glycomacropeptide (GMP), lactoferrin and small peptide fragments are not (usually) recovered (Deshler, 1999). Thus, there are nutritional and possibly functional differences between these two types of WPI.

The proteins of whey represent about 20% of total milk proteins. Whey proteins are globular proteins and are mainly composed of β –lactoglobulin ($\beta$-Lg), alpha-lactalbumin ($\alpha$-La), bovine serum albumin (BSA)**,** immunoglobulins (Ig), minor proteins and enzymes. In whey, about 52% of the protein is $\beta$-Lg and 20% is $\alpha$-La. The distribution and characteristics of whey proteins are shown in Table 1.1. $\beta$-Lg belongs to the lipocalin family of proteins, which is folded as an eight-stranded antiparallel $\beta$-barrel that forms around a central cavity, the calyx. Like most lipocalins, the central cavity of $\beta$-Lg can bind small hydrophobic molecules such as retinol and short-chain fatty acids (Brownlow et al., 1997).

$\beta$-Lg consists of 162 amino acid stabilized by two disulfide bonds: Cys66-Cys160 and Cys106-Cys119. By analogy with the free radical addition polymerization reaction, the exposed thiol-group, Cys121, is able to initiate intermolecular thiol/disulphide interchange reaction. The exposed thiol group is able to form intermolecular disulfide bonds by thiol-oxidation reactions (Roefs & de Kruif, 1994).

Table 1.1. Distribution and characteristics of whey proteins.

| Component | Approximate concentration in milk (g/l) | MW | Groups per mole | | |
|---|---|---|---|---|---|
| | | | pI | -S-S- | -SH |
| $\beta$-Lg | 3.6 | 18,300 | 5.3 | 2 | 1 |
| $\alpha$-La | 1.7 | 14,200 | 5.1 | 4 | 0 |
| Ig | 0.6 | | 4.6-6.0 | Present | |
| IgG1,IgG2 | | 160,000 | (monomer) | and | |
| IgM | | 900,000 | (pentamer) | variable | |
| IgA | | 400,000 | (dimer) | | |
| BSA | 0.4 | 69,000 | 4.7 | 17 | 1 |
| Proteose-peptone | 0.7 | 4,000-40,000 | 3.7 | 0 | 0 |

This model is supported by Hoffmann and van Mil (1997) who reported that once the thiol group of the cysteine residue in $\beta$-Lg was blocked by N-ethylmaleimide (NEM), the polymerization after heating did not occur. It has been also reported that NEM-treated protein molecule after heating was the same as before heating. Further evidence of the importance of the free thiol group is the fact that no gelation occurred for porcine $\beta$-Lg, which lacks the cysteine residue present in the bovine variant (Gallagher et al., 1996). Moreover, Burova et al. (2002) reported that the heat-denaturation of porcine $\beta$-Lg is reversible due to the absence of cysteine residue.

α-La is an acidic, monomeric, calcium-biding metallo-protein. The sequence and teritiary folding of α-La is homologous to that of proteins of the lysozyme family. α-La exerts a significant biological function by participating as a “modifier” protein in lactose synthesis by regulation of the activity of the enzyme galactosyltransferase

(Brodbeck et al., 1967). Its most characteristic physical property is the tendency to undergo time-dependent association at pH values below its isoelectric region. At the pH of milk (~6.6) and above, α-La exists essentially as a monomer. The binding of calcium to α-La causes pronounced changes in its tertiary structure. Removal of calcium reduces the heat stability of the protein. The protein consists of 123 amino acids including cystines. Some studies have shown that α-La on its own does not form aggregates upon heating due to thiol group lacking (Dalgleish et al., 1997; Rojas et al., 1997). The presence of thiol-containing proteins, such as $\beta$-Lg or BSA are prerequisite to induce aggregation, which is in line with the free radical addition polymerization model (Roefs & de Kruif, 1994).

### 1.2. Functional properties and modifications of whey proteins

Whey protein products are often used food ingredients because of their versatile functional and nutritional properties. Their desirable functional properties such as solubility, foaming, emulsification, heat-induced gelation and coagulation, water binding and retention, dispersability, viscosity and turbidity have been primarily revealed and utilized in the food systems (de Wit & Klarenbeek 1984; Morr & Ha, 1993). The manufacture, properties, and uses of whey proteins have been reviewed (Bottomley et al., 1990; de Wit, 1998; Kinsella & Whitehead, 1989; Morr & Ha, 1993). The properties of whey based protein products are mainly dependent on their processing technology. Several different treatments including heat treatments and membrane fractionation techniques, have significant influence on their properties and consequently on their possible use.

The functional behavior of whey proteins during food processing, however, is much more complicated. The native proteins reflect a number of functional properties in aqueous solutions which are modified during processing to affect the protein

functionality. Therefore, the functional properties of protein ingredients are the result of intrinsic properties of whey proteins and a number of extrinsic factors. Intrinsic factors include amino acid composition and sequence, conformation, molecular size, net charge, inter- and intra- cross-links, hydrophilic/hydrophobic ratio, and rigidity/flexibility of the protein in response to external conditions. The relationship between intrinsic properties of whey proteins and extrinsic factors such as temperature, pH, salts, and protein concentration are critically important for elucidating and controlling the functional properties of whey proteins (de Wit, 1998).

Whey protein ingredients have relatively low molecular weight and are able to expose hydrophobic groups when partially unfolded. So, they have the ability to quickly migrate to and adsorb on air-water interface, reducing surface tension, and allowing them to form stable foams (Philips & Kinsella, 1990). This property is usually identified with the foaming ability of whey protein products in aqueous solution (de Wit, 1998). In contrast, whey protein aggregates and polymers impair foaming properties. An important aspect of whey proteins is their success as emulsifiers in food systems. Important factors determining their emulsification properties are protein concentration, pH, ionic strength, concentration of calcium and lactose, the processing history, and the storage conditions (Mccrae et al., 1999). The good emulsifying properties of whey proteins allow the introduction of fat globules as structural elements of heat-induced whey protein gels. Moreover, the well-known heat-induced interactions between whey proteins and casein micelles make milk an interesting base for all kinds of textured products with high nutritional value (de Wit, 1998).

Modification of whey proteins to enhance or alter their functional properties can be accomplished by chemical, enzymatic, or physical techniques (Kester & Richardson, 1984). Chemical modification alters the noncovalent forces (van der

Waals forces, electrostatic interactions, hydrophobic interactions, and hydrogen bonds) determining protein conformation in a manner that results in desired structural and functional changes. The modification of whey proteins by sulfitolysis was shown to improve their emulsion activity index, viscosity, foaming capacity and foam stability and digestibility of $\beta$-Lg (Kella et al., 1989; Klemaszewski & Kinsella, 1991). Enzymatic modification generally involves proteolytic hydrolysis of the protein to yield a mixture of peptides. Functional behavior usually is altered in a manner dependent upon the extent of hydrolysis. Enzymes also can be used to introduce intramolecular or intermolecular crosslinks into a protein structure (Eissa & Khan, 2006). Such chemical crosslinks bring favorable textural and rheological properties increasing the elasticity of whey protein gels (Dickinson, 1997). Physical protein modification may involve thermal treatment, complex formation with biopolymers, or a texturization process. Thermal treatment resulting in partial protein denaturation may elicit desired improvements of functional behavior. Complete denaturation, however, usually results in a loss of solubility and other functional properties. Texturization of protein involves physical treatments such as fiber spinning or thermoplastic extrusion. These processes impart structural integrity to proteins (Kester & Richardson, 1984).

### 1.3. Denaturation and aggregation of whey proteins

The behavior of whey proteins during food processing is very complex and is governed by their heat sensitivity (de Wit & Klarenbeek, 1984) or they depend not only on their intrinsic properties but also on their susceptibility to denaturation. Denaturation has been defined as a major change of the very specific native structure, without alteration of the amino acid sequence and is a consequence of an altered balance between the different forces, such as electrostatic interactions, hydrogen bonds, disulfide bonds, dipole-dipole interactions, and hydrophobic interactions that

maintain a protein in its native state. Therefore, denaturation of globular proteins is in most cases a prerequisite to “activate” the functionality that is desired for the sensorial and textural properties of food. The functional properties of whey proteins such as foaming and emulsifying properties can be altered by heating under defined conditions.

$\beta$-Lg, the major protein in whey, tends to dominate the thermal behavior of the total protein system (Hoffmann & van Mil, 1999). It is predominantly present as a dimer in aqueous solutions at pH values between 5.5 and 7.5 (McKenzie & Sawyer, 1967). Several studies indicated that the initiation of the polymerization reaction involves dissociation of the dimers, followed by a critical change in the confirmation of $\beta$-lg at 60-65 ºC, which exposes the buried thiol group of Cys121 on the protein surface and become reactive (Verheul et al., 1998, 1999). The dimer dissociation step was shown to be a necessary step in heat-induced aggregation mechanism (Cairoli et al., 1994; Iametti et al., 1996). Then, the free thiol group attacks an intra-molecular disulfide bridge in another protein monomer and reacts with it through an exchange reaction or it initiates sulfhydryl/disulfide interchange reactions called propagation step. A new intermolecular disulfide bridge is formed and a new free reactive thiol group for the propagation step is produced. The propagation step proceeds until termination occurs when two free reactive thiol group react with each other creating a new disulfide bond but no free thiol group, leading to irreversible aggregation/polymerization (Roefs & de Kruif, 1994). The primary aggregates may form larger secondary aggregates built up from chemical and physical interactions, i.e. disulfide bridges, electrostatic forces, hydrogen bonds and hydrophobic forces. These results were extended and it was shown that blocking the thiol group gave a protein derivative that would not aggregate via disulphide interchange reactions (Hoffmann & van Mil, 1997; Iametti et al., 1996; Sawyer, 1968; Verheul et al., 1998).

The above whey protein aggregation kinetic can be well described when $\beta$-Lg is in the temperature range of 60-70 ºC and at near-neutral pH (Roefs & de Kruif, 1994). However, this assumption may not hold at other pH values, leading to different overall kinetics. Roles of the free thiol group and disulfide bonds as well as the effect of pH in the range 6.0-8.0 on the denaturation and aggregation of $\beta$-Lg were investigated by Hoffmann and van Mil (1997, 1999). The results revealed that the rate conversion of native $\beta$-Lg increased strongly at higher pH values, whereas the molecular mass of the aggregates decreased strongly. Tanford et al. (1959) described that $\beta$-Lg undergoes a reversible change of conformation between pH 6 and 8.5. At lower pH, the N-state prevails, and in this conformation the thiol groups are buried in the $\beta$-Lg dimers, presumably in the region of contact between the monomer subunits. At higher pH, where the R-state prevails, a refolding of the protein chains appears, resulting in a higher reactivity of the thiol group. Therefore, at lower pH, the molecule has to be heated, or unfolded in another way, in order to expose the thiol group for reaction. Moreover, McSwiney et al. (1994) observed an increase in the rate of polymerization via sulfhydryl/disulfide exchange reactions as the pH was increased from 6.0 to 9.0 due to increased accessibility and deprotonation of the thiol group at higher pH values. It was concluded that the pH dependence reflected the midpoint unfolding temperature and not the thiol group reactivity, suggesting that this reactivity was not rate limiting in the aggregation.

### 1.4. Gelation of whey proteins

Whey proteins have been typically used in food formulations for their ability to gel on heating and provide textural properties which is very important to consumer acceptability. Heat- induced gelation of whey proteins have been extensively studied and reported (Mangino, 1992; Mulvihill & Kinsella, 1987; Ziegler & Foegeding,

1990). The ability of protein solutions to form self-supporting gels depends on a favorable balance of attractive and repulsive forces between protein molecules. Both non-covalent (e.g., H-bonding, hydrophobic, and electrostatic interactions) and covalent (e.g., disulfide bonds) forces drive the gelation process. There are factors that affect protein gelation, as well as affecting the type and properties of gels (Hermansson, 1979; Kinsella et al., 1994) can be classified, according to Philips et al. (1994), as intrinsic and extrinsic, as listed in Table 1.2.

Table 1.2. Classification of interaction in protein gel formation (Philips et al., 1994).

| Intrinsic factors | Extrinsic factors |
|---|---|
| Hydrophobicity | Protein concentration |
| Electrostatic Interactions | pH |
| Disulfide bonds | Temperature |
| Molecular weight | Ionic strength and type of Ion |
| Amino acid composition | Pressure |

### 1.4.1. Heat-induced gelation

Most food protein gels are formed during heating and are therefore referred to heat-induced or heat-set gel. The thermal gelation is a two a two-stage process involving an initial unfolding and subsequent aggregation of protein molecules as shown in Figure 1.1 (Alting, 2003; Verheul et al., 1998; de Wit & Klarenbeek, 1984). The texture and gel strength are affected by intrinsic factors and also by extrinsic factors (Damodaran, 1989; Foegeding, 1989; Schmidt, 1981). Alteration of heat treatment conditions (temperature and time) affect the gel's macroscopic and microscopic structural attributes (Schmidt, 1981), by changing the rates and mechanisms of denaturation and aggregation. Boye et al. (1995) concluded that WPC

formed firm gels at temperature above 70 ºC, at alkaline pH range and in the absence of NaCl.


Figure 1.1. Conversion of native globular protein into a protein network during heat-induced gelation (Alting, 2003).

When whey protein solutions were heated, $\alpha$-La did not aggregate above pH 7 but denatured readily to form aggregates at acid pH while $\beta$-Lg would aggregate at both acid and alkaline pH regions. However, whey proteins denature completely on heating at 90 °C for 10 minutes. $\beta$-Lg is reported to be the most heat-labile among the whey proteins while $\alpha$-La is considered the most heat-stable because of its ability to renature below 65 °C (Morr & Ha, 1993).

In the case of samples that form gels, the conditions of pH and temperature are sufficient to overcome the stabilizing forces which prevent the native proteins from interacting to form a three-dimensional gel network. However, enhancing one type of interaction may not be sufficient to overcome other forces that collectively prevent gelation. For example, while extensive disulfide bond formation is evident in WPI at pH 9 and samples are visibly more viscous compared to the unheated WPI at pH 7, the overall interactions are insufficient for the proteins to form a gel network (Monahan et al., 1995).

Native whey proteins can easily form aggregates in acidic and salt environments or by proteolysis at temperature far below the denaturation temperatures of proteins (20-45 ºC). Therefore, the pH and salt concentration of protein dispersions

have a pronounced effect on gelation, by influencing the balance of polar and non-polar residues (Boye et al., 1995). Under appropriate ionic strength, pH, and thermal gelation conditions, whey protein gels are capable of immobilizing large quantities of water and other ingredients. Adjustment of these physical conditions determines the structural network, water holding, and rheological properties possessed by the gels. At low ionic strength or at pH values far from the iso-electric point (pI) of the active protein, electrostatic repulsive forces hinder the formation of random aggregates and more linear polymer are formed, resulting in transparent and fine-stranded gels, and having a good water holding capacity. When heat-induced gelation occurs at high ionic strength or at pH near the pI of the protein, repulsive forces are weaker and denatured proteins aggregate randomly by physical interactions such as hydrophobic and van der Waals interactions into particulate, turbid gels. Particulate gels have generally lower water holding capacity because of the large inter-particle pores (Elofsson et al., 1997).

### 1.4.2. Cold-gelation

In addition to heat-induced gelation, other gelation methods of whey proteins are reported such as salt-induced gelation, acid-induced gelation, and enzyme-induced gelation (Totosaus et al., 2002). Salt- or acid-induced types of gelation consist of two steps. The gelation step, induced by the addition of salt or acid, has to be preceded by an activation step in which the protein molecule denatures and forms soluble protein aggregates. In the literature this process is known as “cold gelation of globular proteins”. Cold gelation of heated protein solutions has been reported for $\beta$-Lg, WPC, and WPI (Barbut & Foegeding, 1993; Sato et al., 1995; Elofsson et al., 1997; Ju & Kilara, 1998a,b; Vreeker et al., 1992).

Ju and Kilara (1998a) reported that additions of $CaCl_2$ (10-40 mM), NaCl (50-400 mM) or hydrolysis by a protease from *Bacillus licheniformis* caused gelation of the denatured whey protein solution at 45 ºC. Their results revealed that glucono-δ-lactone (GDL)-induced gels were harder than salt-induced gels, and much harder than the protease-induced gel. The gelation required addition of minimum salt concentrations (>5 mM $CaCl_2$ and > 20 mM NaCl) or a resultant low pH (<5.8). They also found that maximum gel hardness occurred at 200 mM NaCl or pH 4.7, and increasing $CaCl_2$ concentration (up to 40 mM) continuously increased gel hardness. Barbut and Foegeding (1993) also reported that increased $CaCl_2$ concentration (10-150 mM) progressively increased shear stress of $CaCl_2$-induced gel.

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. In the second step, it is possible to study how the properties of the aggregates influence the gelation process. However, control of the gelation process by modification of the aggregates after the heating step has not been reported yet. It appears that disulfide bonds are formed in the second stage of the process of cold gelation and that they have an influence on the final mechanical properties of the gels. It was shown that formation of disulfide bonds increased the MW of the aggregates formed during gelation, and these bonds were involved in stabilizing the network, resulting in a much stronger gel (Alting et al., 2000).

A typical acid-induced cold-set gel is formed by a gradual and slow acidification of the solution of protein-aggregates by addition of GDL. In aqueous solutions this component slowly hydrolyzes to gluconic acid, causing a gradual lowering of the pH. The role of electrostatic interactions has been demonstrated in the

past by the addition of salts to shield the electric charge of the proteins and by studying the pH-dependency of gelation (Matsudomi et al., 1991; Wang & Damadoran, 1991). These approaches do not only result in a change of the net charge of the protein, but also have potential side-effects such as a promotion of hydrophobic interactions. With respect to the formation of intermolecular disulfide bonds, Alting et al. (2000) demonstrated the importance of these interactions for the mechanical properties of cold-set, acid-induced gels of WPI. The formation of disulfide bonds predominantly occurs under alkaline conditions. They also have shown that formation of disulfide bonds also occurs in acid-induced cold-set gels at pH 5.

Several authors (Alting, 2003; Barbut & Foegeding, 1993; Hongsprabhas & Barbut, 1996; McClements & Keogh, 1995; Sato et al., 1995) reported cold gelation of whey proteins. These ingredients have been formed by two stage process 1) preparation of a heat-denatured whey protein solution, and 2) induction of gelation at low temperature by lowering the pH or by adding salt. However, they did not describe a dry powder for cold-set whey protein gelation. In theses studies, whey protein solutions (6-10% w/v), of very low ionic strengths at pH 7, were heated at 70-90 ºC and cooled quickly to room temperature that no gel was formed. After cooling, a stable dispersion of aggregates is obtained. In the second step, gelation can be induced at ambient temperature by addition of NaCl (McClements & Keogh, 1995), $CaCl_2$ (Barbut & Foegeding, 1993; Hongsprabhas & barbut, 1996; Nakamura et al., 1995) or by a decrease in pH (Kawamura et al., 1993).

Other studies were conducted on cold-gelling whey proteins in dried form. Thomsen (1994) described that this whey protein ingredient was produced by thermal treatment during homogenization of WPC at slightly alkaline pH followed by immediate drying process. This product is commercially available as the modified WPC powder with potential application in food such as surimi, comminuted meats,

dressings, and bakery products. This ingredient can form gel upon reconstitution in a salt solution. Keogh (1998) also reported the production of dried, denatured, whey protein-based powders, which on reconstitution in food formulations show an increased ability to bind water in the presence of added salts, especially in the ambient temperature range. The results showed that a preheated whey protein ingredient improved the consistency of surimi and a cold-set dessert system. The pre-heated whey protein dispersions are also capable of binding and stabilizing calcium phosphate. This property can be exploited in the stabilization of calcium-fortified milk-based beverages.

Hudson et al. (2000) and Resch and Daubert (2002) developed a derivatization process of WPI and WPC in an attempt to imitate the similar functional properties of pre-gelatinized starches. WPC or WPI solution (12% w/v) was adjusted to the pH 3.35 with 6N HCl or 6N NaOH, heated (80 ºC) to a gel, freezed dried, and grounded into powder. Their results showed that 10 % (w/w) protein dispersion exhibited the shear thinning behavior and this product was able to form cold set weak gel and contribute viscosity in food systems. Consequently, Daubert et al. (2004) were able to produce derivatized whey powders with a more economical process that replaced the freeze drying with a spray drying. They found that at concentrations of 5-13%, the spray dried WPC samples exhibited pseudoplastics behavior. Also, at equal concentrations, the spray dried whey proteins were more viscous than the freeze dried products.

Because cold gelation can occur after addition to a food matrix, it has considerable potential in the food industry. The use of whey proteins or other industrial proteins without the need to heat the final products is an attractive alternative for current thickening ingredients (Bryant & McClements, 1998). The mechanical properties of both cold and heat-set gels depend on the protein

composition and concentration, their interaction with other ingredients, and the preparation technique.

### 1.5. Texturization of whey proteins by extrusion process

The most commonly techniques used to texturize whey proteins is thermoplastic extrusion (Martinez-Serna & Villota, 1992). Extrusion cooking has been applied to dairy ingredients to improve functional properties, replace the traditional by continuous process, and develops foods with new texture characteristics. Extrusion with their shearing screws operating at varying speed and heating can alter the conformational structure of globular proteins changing the molecular structure of proteins and forming new functionalities (Martinez-Serna & Villota, 1992). The early reason for texturizing protein is to develop a physical structure which will provide, when eaten, a sensation of eating meat. Textured proteins can broaden the range of food applications to include use as meat analogues or meat extender. In these cases, the structure of the protein must be made to resemble that of muscle in order to attain the proper texture. Whey protein has been considered as the new ingredient for meat alternative market and snack because it is readily available, inexpensive, and high protein source (Onwulata et al., 2003; Walsh & Carpenter, 2003).

Hale et al. (2002) developed meat extender for beef patties by extruding 2 parts of WPC and 1 part of corn starch using water, 0.1 N HCL, or 0.2 M NaOH as the liquid. The sensory results showed that consumers identified no difference in taste or texture between burgers made using 40% base texturized whey proteins and 100% beef. Singh et al. (1991) reported that adding non-texturized whey proteins as a co-extrudate ingredient for the extrusion process of puffed products did not improve the expansion and crunchiness of the extrudates. Kim and Maga (1988) also indicated that blends of extruded WPC and starch flours (potato, rice, or corn flour) had lower

expansion ratio and water absorption than the starch extrudate. However, a sensory panel preferred rice extruded with WPC to rice without it. Therefore, effort is needed to improve whey protein functionality by texturizing whey protein before combining with other components such as starches, flours, hydrocolloids, and other nondairy proteins. Onwulata et al. (2003) studied the functionality of texturized whey proteins by extruding three different types of whey protein including WPC, WPI, and whey albumin at 38% moisture content at different cooking temperatures. They found that varying temperature in the extruder demonstrated the different degree of whey protein denaturation which might be useful for different products.

Walsh and Carpenter (2003) developed a new snack product by extruding a dry mix comprising 2 parts of WPC80 and 1 part of corn starch at a rate of 25 g/min and adding 0.1 M NaOH solution at a rate of 11 g/min at 145-147 ºC. The resulting product was an expanded, crunchy with small even cell size. Recently, Onwulata et al. (2006) extruded WPI pastes (60% solids) in a twin screw extruder at 100 ºC with four different pH adjusted water streams; acidic solutions (pH 2.0 and 2.5), and alkaline solutions (pH 11.5 and 12.4). The results indicated that alkaline treatment increased insolubility and pasting properties (viscosity). This condition also produced rod-like microstructures and formed fine-stranded fiber-like structures in texturized products. Acidic conditions increased solubility and decreased WPI pasting properties.

An extruder can also serve as a continuous chemical reactor. Barraquio et al. (1988) reported the acid coagulation of skim milk powder in a twin screw extruder at 25-35% moisture content and cooking temperature of 50-94 ºC. Extrusion cooking has been suggested for lactose hydrolysis, and also for the preparation of sugar-milk protein confection (Jones, 1989). However, extrusion cooking at high moisture content can be used also to produce cheese analogs using a long barrel (1500 mm) (Zuber et al., 1987; Cavalier et al., 1990). Queguiner et al. (1992) studied microcoagulated

protein prepared from a WPI rich in $\beta$-Lg and containing less than 1% fat or lactose. Protein coagulation was carried out at acid pH, high protein concentration (15-25%), a barrel temperature of 90-100 ºC, pH range 3.5-3.9, and a screw speed of 100-200 rpm. The results showed that semi-solid spread (pH 3.9) were obtained, and composed of small coagulated particles with mean diameter of 11.5 µm (volume basis). They also suggested that this extrusion-coagulated WPI can be used as fat substitute in food systems to assess creaminess-inducing properties.

### 1.6. Molecular transformations of proteins during extrusion process

The structure formation of protein extrudates is believed to result from a complete restructuring of the polymeric material in an oriented pattern (Kinsella, 1978). The forces which stabilize the tertiary and quaternary structures of the proteins are weakened by a combination of increased temperature and shear within the extruder (Camire, 1990). During extrusion, the proteins completely disaggregate through mechanical mixing to form a homogeneous suspension. Consequently, the proteins are denatured, dissociated, and unraveled, allowing alignment of the denatured protein molecules in the direction of the flow (Li & Lee, 1996). The reaction sequence is depicted in Figure 1.2. The proteins then cross-link at the die end of the extruder to impart a network to the extrudates (Martinez-Serna & Villota, 1992). However, the way that protein cross-links with protein in the extrusion process is still unclear, and no unified model or mechanism for protein−protein interactions during extrusion processing has been proposed to date.

Several reports on protein interactions in soy extrusion claimed that disulfide bonds were of negligible importance in the final structure of extrudates, suggesting that new peptide bonds formed in the severe conditions of extrusion (about 180 °C) were responsible for the structure of these products (Burgess & Stanley, 1976;

Simonsky & Stanley, 1982; Stanley, 1986). These results were based on the detection of an increase of the free sulfhydryl content after soy extrusion and on the decrease in texture formation after blocking free amino and carboxyl groups of proteins with ninhydrin and citric acid, respectively. However, this proposed mechanism has been widely disputed. When extruded soy and whey proteins were solubilized in reagents which exhibited specific chemical actions on proteins (disrupting hydrophobic and electrostatic interactions, hydrogen bonds, and disulfide bonds), their resolubilized profiles indicated that protein interactions resulted primarily from the disulfide bonds formed from cysteine residues and, less importantly, nonspecific hydrophobic and electrostatic interactions (Hager, 1984; Jeunink & Cheftel, 1979; Arêas, 1992; Martinez-Serna & Villota, 1992; Prudêncio-Ferreira & Arêas, 1993). Supporting this finding, Strecker et al. (1995) have shown that with a small increase in disulfide bond formation a large increase in network formation resulted in wheat proteins, which provided insight into the extent that disulfide bonds contributed to the polymerization of wheat proteins. So far, it is still unclear what the overall mechanism of protein interaction is during the extrusion process, which has prevented the application of the extrusion process in improving functional and textural properties of underutilized protein sources.

### 1.7. Supercritical fluid extrusion

Supercritical fluid extrusion (SCFX), a novel extrusion technology for production of highly expanded starch foam, was patented by Rizvi and Mulvaney (1992). Instead of steam this process uses supercritical $CO_2$ (SC-$CO_2$) as a blowing agent, a nutrient carrier, and as an in-line process modifier (Alavi et al., 1999). SC-$CO_2$ is an environmentally friendly solvent, chemically inert, physiologically safe, easily recycled which is ideal for food processing (McHugh & Krukonis, 1994).

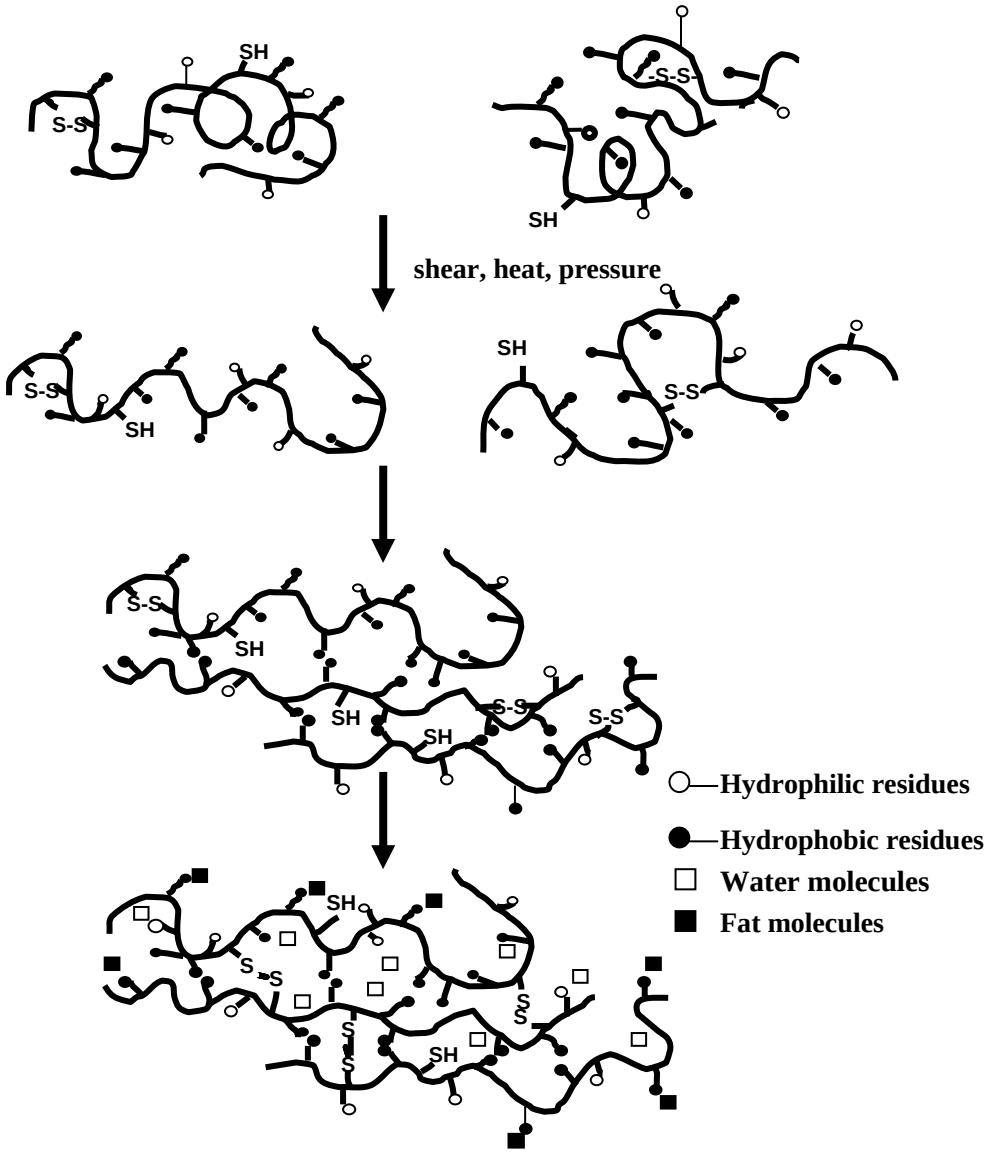


Figure 1.2. Schematic diagram of a protein molecule denaturing, aligning in the direction of flow and cross-linking through hydrophobic interactions and disulfide bond formations with another protein during extrusion processing (Li & Lee, 1996).

Thermodynamically, SC-$CO_2$ has a liquid-like density and gas-like diffusivity and viscosity which leads to rapid wetting and allows penetration of complex structure (Rizvi et al., 1995). The supercritical conditions of $CO_2$ are relatively easy to achieve (critical temperature = 31˚C, critical pressure = 7.38 MPa). SCFX is conducted at high

pressure and at temperatures lower than 100°C with lower shear which offers major advantage over to steam-based extrusion processing. The potential of using SCFX for producing a range of puffed food products such as ready-to-eat cereals, pasta, and confectionery products has been reviewed (Rizvi & Mulvaney, 1992). Its distinct low-temperature and low-shear conditions due to high moisture allow for the retention of heat sensitive ingredients. The delicate balance of temperature, pressure, and shear and internal environment created by introduction of SC-$CO_2$ during SCFX processing creates opportunities for chemical reactions and conformational changes in proteins.

SCFX process dynamics can be divided into two stages- I) flow of protein melts containing SC-$CO_2$ through the nozzle and extruder die, II) exit of extrudate from the die. At a macroscopic level, the predominant phenomena included pressure drop experienced by protein melt in stage I, bulk diffusion of $CO_2$ and heat transfer in stage II. At the microscopic level, the predominant phenomena include nucleation of bubbles as the protein melt saturated with SC-$CO_2$ undergoes a pressure drop in stage I, expansion of the individual bubble induced by a net driving force acting upon the surrounding protein matrix and diffusion of $CO_2$ from protein matrix into the bubble in stage II. Therefore, the expansion process consists of three steps- a) dissolving SC-$CO_2$ in the polymer melt to form a polymer and SC-$CO_2$ solution, b) cell nucleation caused by rapid pressure drop and c) cell growth and extrudate expansion at the die exit as the pressure quenches to atmospheric level (Alavi et al., 1999; Mulvaney & Rizvi 1993; Rizvi et al., 1995).

The SCFX process is a more versatile and controllable. In this process, the pressure drop can be manipulated by adjusting the operating conditions, therefore the cell size, cell density, and product expansion can be varied to produce a wide range of products having desired mechanical properties.

### 1.8. Rational and significance

Whey proteins are often used in a variety of food formulations due to their unsurpassed nutritional quality and inherent functional properties. The possibilities for the improvement and upgrading of whey protein utilization still need to be explored. Intensive research efforts on food proteins are aimed at modifying inexpensive, available proteins to enhance their functionalities so that more costly proteins in the formulation could be spared. The importance of correlating protein functionality to structure is a first step in a more systematic approach to understanding and designing processes to improve, as well as predict, functionality. However, the functional properties of proteins are the result of intrinsic properties of proteins and a number of extrinsic factors. Intrinsic factors include amino acid composition and sequence, conformation, molecular size, net charge, inter- and intra- cross-links, hydrophilic/hydrophobic ratio, and rigidity/flexibility of the protein in response to external conditions. The relationship between intrinsic properties of proteins and extrinsic factors such as temperature, pH, salts, and protein concentration has been known to be critically important for elucidating and controlling the functional properties of proteins (de Wit, 1998). Many successful protein functionality augmentations have been achieved by chemical, enzymatic, or physical techniques (Kester & Richardson, 1984). Among the physical methods, protein functionality modification by cooking extrusion has received considerable attention as a means for texturization of proteins and the use of twin-screw extrusion has proven to be instrumental in the development of many new products. Until now, only a few investigators have addressed the mechanisms of protein functionality and reactivity changes during extrusion, especially at high protein concentrations and high water content (Onwulata & Tomasula, 2004; Stanley, 1986; Walsh & Carpenter, 2001). This study has been conducted based on the knowledge that the combination of shear,

temperature and pressure during high-pressure reactive extrusion processing can creates opportunities for both conformational changes and chemical reactions in proteins and form new functionalities. The high pressure extrusion process of proteins could be achieved by introduction of dense carbon dioxide. Utilization of dense carbon dioxide in the extruder can decouple the two roles of water in the conventional extrusion cooking processes, i.e., where it plays the role of both a plasticizer and a blowing agent in making expanded extrudates. Expanded extrudates can thus be made at sub 100 $^{0}$C temperatures, which obviates the need for high temperature treatment of heat sensitive proteins and also provides a precise control of the extent of denaturation and reactivity achieved.

Texturization of protein by conventional cooking extrusion is a currently practiced industrial technology. While it is known that the product's texture results from a complete restructuring of the polymeric material into an oriented pattern followed by cross-linking at the die end of the extruder (Kinsella, 1978; Martinez-Serna & Villota, 1992), the nature of the interactions among the various other processing parameters like the ionic strength, the pH and pressure obtained via injected carbon dioxide, shear rate, and temperature are not known. For this research, a twin-screw extruder is used as a continuous bioreactor to generate microcellular extrudates by precisely controlling the above variables. The proposed texturization process of whey proteins by reactive SCFX is shown in Figure 1.3.

### 1.9. Scope of the study

This research focuses on developing the basic and applicable information on the effects of the superimposed process variables such as shear, temperature, carbon dioxide pressure, pH, and ionic strength on the physicochemical properties of commercially available WPC-80. The scientific data, technological approaches and

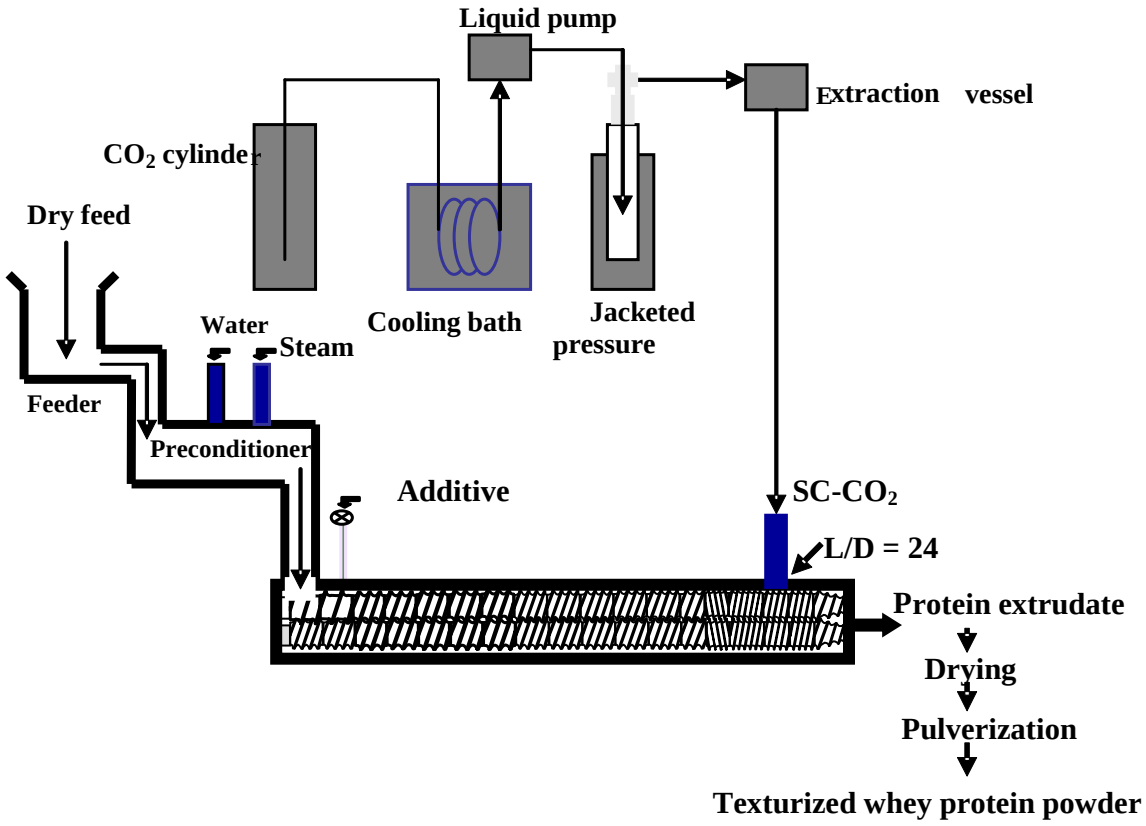


Figure 1.3. Schematic for production of texturized whey proteins by reactive supercritical fluid extrusion (RSCFX).

prototypes to allow the development of texturized whey proteins (tWPC) using a novel reactive supercritical fluid extrusion process will be established. The unique advantage of high-pressure reactive extrusion and the possibility to determine and to manipulate conformational structure and functional properties of proteins is exploited to identify the key factors that regulate this process. Therefore, the ultimate goal of this research is to investigate and establish sufficiently fundamental understanding of the reinforcement mechanism and processing of whey protein texturization, to facilitate

development of a unique protein product having a wide rage of improved functional properties.

In this thesis, the chapters are organized in the following manner:

- **Chapter One**: In this chapter, a brief overview on whey protein functionalities and modifications, gelations, texturization, and transformations of proteins during extrusion process is presented. The concept of SCFX process is also included.
- **Chapter Two**: In this chapter, the influence of pH conditions and SC-$CO_2$ injection during reactive SCFX on the rheological behaviors of tWPC powders upon reconstitution with water at ambient temperature was investigated. The performance of thickening and gelling of tWPC powders was evaluated using a range of rheological techniques. The ability of tWPC powders to impart instantaneous thickening and gelling functionality over a range of temperature was also investigated.
- **Chapter Three**: In this chapter, the mechanisms of interactions of proteins during reactive SCFX in highly acidic (pH 2.89) and alkaline (8.16) conditions and their influences on the selected rheological and physicochemical properties of the tWPC products were investigated. The contribution of covalent (disulfide bonds) and non-covalent (hydrophobic and hydrogen bonds) interactions in tWPC samples were evaluated and compared with the extruded (non-pH-adjusted) and the unextruded controls on the basis of protein solubility in different extraction solutions, SDS-PAGE, and sulfhydryl (SH) group content, protein aggregate size, and apparent viscosity.
- **Chapter Four**: In this chapter, the emulsifying activity and emulsion activity of tWPC were evaluated and compared with the commercial WPC80. The thermal stability and the effects of oil fractions (20-80%, w/w) on the

rheological properties of gel-like emulsions prepared with tWPC and commercial WPC80 was also investigated.

- **Chapter Five**: In this chapter, the effect of tWPC on the stability of emulsions containing 20-80% (w/w) butter oil (crystallizable oil) and corn oil was examined. The effect of storage temperatures on the stability of tWPC-stabilized emulsions was investigated. The stability of emulsions was assessed by phase separation, droplet size distribution and morphology, and rheological characterization.

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# CHAPTER TWO

# RHEOLOGICAL CHARACTERIZATIONS OF TEXTURIZED WHEY PROTEIN CONCENTRATE-BASED POWDERS PRODUCED BY REACTIVE SUPERCRITICAL FLUID EXTRUSION*

## 2.1. Abstract

A powder blend comprising (by weight) 94% whey protein concentrate (WPC-80), 6% pre-gelatinized corn starch, 0.6% $CaCl_2$, and 0.6% NaCl was texturized using a supercritical fluid extrusion (SCFX) process. The blend was extruded at 90 °C in a pH range of 2.89-8.16 with 1% (db) supercritical carbon dioxide (SC-$CO_2$) and 60% moisture content. The texturized WPC-based (tWPC) samples were dried, grounded into powder, reconstituted in water, and evaluated using a range of rheological studies. Most tWPC samples exhibited shear thinning behavior and their mechanical spectra were typical of weak gel characteristics. The tWPC produced under extremely acidic condition of pH 2.89 with SC-$CO_2$ yielded the highest $\eta^*$ (10,049 Pa s) and $G'$ (9,885 Pa) compared to the unprocessed WPC ($\eta^*$=0.083 Pa s and $G'$=0.036 Pa). The SCFX process rendered WPC into a product with cold-setting gel characteristics that may be suitable for use as a food texturizer over a wide range of temperatures.

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## 2.2. Introduction

The development of new approaches through use of novel technologies to better utilize food ingredients in general and dairy ingredients in particular is a major challenge in today's competitive world. Whey proteins (WPs) are widely used in a variety of food formulations and constitute a significant share of the dairy ingredients market. Their ability to gel upon heating provides desirable food textures which are important to consumer acceptability.

The most common technique used to texturize WPs is thermoplastic extrusion (Martinez-Serna & Villota, 1992). This technique provides thermal and mechanical energy for mixing, cooking, melting, and forming biomaterials with varied functional characteristics. The extrusion process has been applied to dairy ingredients to improve functional properties, replacing the traditional technique with a continuous process, and develop food products with new textural characteristics. The texturization of whey protein concentrate (WPC), whey protein isolate (WPI), and nonfat dry milk through a twin-screw extruder has been reported to develop ingredients for possible uses as fat substitutes (Queguiner, Dumay, Salou-Cavalier, & Cheftel, 1992), meat extenders (Hale, Carpenter, & Walsh, 2000; Walsh & Carpenter, 2003), high protein snacks, and protein-fortified foods (Michael & Onwulata, 2006; Walsh & Carpenter, 2003).

In general, functionality of WPs can be altered by heating, adding salts, adjusting the pH, and shearing. Heat-induced gelation of WPs has been extensively studied and used to add texture to food products (Mangino, 1992; Mulvihill & Kinsella, 1987; Paulsson, Hegg, & Castberg, 1986; Ziegler & Foegeding, 1990). However, foods containing WPs had to be heated above 65 ºC before the proteins would form gels or thicken solutions. This limited their application in many types of products containing heat sensitive ingredients. Currently, there is considerable interest in converting WPs into cold-gelling ingredients which can be used as thickening

ingredients in food applications where heating is not desirable to achieve desired viscosities. Cold gelation of heated protein solutions has been reported for β-lactoglobulin (β-Lg), WPI, and WPC (Alting, 2003; Barbut & Foegeding, 1993; Bryant & McClements, 1998; Elofsson, Dejmek, Paulsson, & Burling, 1997; Ju & Kilara, 1998a,b; Sato, Nakamura, Nishiya, Kawanari, & Nakajima, 1995; Vreeker, Hoekstra, den Boer, & Agterof, 1992). Specifically, these cold-gelling WPs have been produced from pre-heated protein dispersions treated with mineral salts addition or pH reduction. The dried form of cold-gelling WP which could form gel upon reconstitution in a salt solution was reported by Thomsen (1994). This product was produced by a heat treatment of WPC solution under a mildly alkaline condition during homogenization followed by a drying process. In addition, the development of freeze-dried and spray-dried derivatized WPI and WPC powders were reported by Hudson, Daubert, and Foegeding (2000), and Resch (2004). These derivatized WP powders were produced by acidifying WPI or WPC solutions (12% protein, w/v) to pH 3.35. The solutions were then heated to 80 ºC for 3 h and freeze dried or spray dried to produce the derivatized, dried gel WP powders. The authors reported the ability of derivatized WP powders in forming a cold-set thickening agent at ambient temperature. Although numerous studies on WP modification have been reported, several barriers still remained. The novel technology approach is needed to open up a new avenue for WP utilization in food formulations.

It is evident that protein unfolding and aggregation are particularly sensitive to pH and ionic strength due to its dependence on electrostatic interactions, resulting in different gel structures. Extrusion with their shearing screws and heating can also alter the conformational structure of globular proteins. This occurs through partial denaturation of protein, thereby exposing the reactive groups that are normally buried in the native proteins (Kim & Maga, 1987; Onwulata, Isobe, Tomasula, & Cooke,

2006; Onwulata, Konstance, Cooke, & Farrell, 2003). It is believed that extrusion processing of WP in the presence of alkali or acid may impart conformational and rheological changes that would lead to new functional properties. However, limited information is available on the functionality and rheological performance of WPs modified by extrusion process. Few investigators have addressed the texturization of WPs by extrusion process for use as cold-gelling agents. In this research, our strategy was to modify WP gelling and functional properties using a novel supercritical fluid extrusion process (SCFX).

SCFX is an innovative food processing technology that offers sub-100ºC expansion using direct supercritical fluid carbon dioxide (SC-$CO_2$) injection. Its distinct low-temperature and low-shear conditions due to high moisture allow for the retention of heat sensitive ingredients. The general expansion mechanism of SCFX process consists of the following major steps: (a) development of gas holding matrix by heat-shear treatment, (b) injection of SC-$CO_2$ into the matrix and mixing in the extruder barrel to create a saturated solution, (c) nucleation of cells induced by thermodynamic instability created by a sudden pressure drop at the die, (d) cell growth and extrudate expansion at the die exit as the pressure quenches to atmospheric level (Alavi, Gogoi, Khan, Bowman, & Rizvi, 1999; Mulvaney & Rizvi, 1993). The delicate balance of temperature and shear during SCFX processing permit controlled modification of WP conformation. The SC-$CO_2$ is an environmentally friendly solvent and is chemically inert which is ideal for food processing. Addition of SC-$CO_2$ provides additional acidic environment and also serves as blowing agent for surface modification of WP matrices. It was hypothesized that reactive SFCX process in highly alkaline or acidic environment combined with controlled shear and heat in the presence of mineral salts ($CaCl_2$ and NaCl) and SC-$CO_2$ would favorably alter gelling and functional properties of WPC.

Rheological measurements have been considered as an analytical tool to provide fundamental insights on the structural organization of materials. The small-amplitude oscillatory (dynamic) tests (SAOS) have been commonly used to characterize the viscoelastic behaviors of food samples and also gel-like materials. It allows researchers to relate dynamic rheological parameters to the molecular structure of materials (Gunasekaran & Ak, 2000). The overall objective of this study was to determine the influence of altering pH and SC-$CO_2$ injection using reactive SCFX process on the rheological behaviors of tWPC-based powders upon reconstitution with water at ambient temperature. The performance of thickening and gelling of tWPC-based powders was evaluated using a range of rheological techniques. The ability of tWPC-based powders to impart instantaneous thickening and gelling functionality over a range of temperature was also investigated.

## 2.3. Materials and methods

### 2.3.1. Materials and feed formulation

Commercial WPC-80 was obtained from Leprino Foods Company (Lemoore West, CA, USA). Pre-gelatinized corn starch (Hammond, IN, USA), NaCl, and $CaCl_2$ (Sigma Chemical Co., St. Louis, MO, USA) were also added to a WPC-based dry mix. The pH-adjusting agents, NaOH and HCl solutions (Sigma Chemical Co., St. Louis, MO, USA), were injected to the extruder at the mixing zone 1 (Figure 2.1). Pre-hydrated (10% dry basis) WPC-80 powder (94%, w/w), pre-gelatinized corn starch (6%, w/w), $CaCl_2$ (0.6% WPC-starch basis, w/w), and NaCl (0.6% WPC-starch basis, w/w) were blended and then preconditioned at ambient temperature overnight before feeding into the extruder.

### 2.3.2. Texturization of WPC by SCFX process

A pilot-scale Wenger TX-52 Magnum (Wenger Manufacturing, Sabetha, KS, USA) co-rotating twin screw extruder was used to texturize WPC-based blend. This extruder with 4.5 heads, a barrel diameter of 52 mm, and a length to diameter ratio (L/D) of 28.5:1 was specially configured for the process. The SCFX process was operated at the screw speed of 180 rpm, product temperature of 90 ºC, and feed rate of 35 kg/h. The die was fitted with two circular inserts of 1.2 mm diameter each. The screw configuration, and temperature control zones for extrusion are illustrated in Figure 2.1. Extrusion was conducted at 60% (dry basis) moisture content. To modify the pH of WP polymer melts in the extrusion process, HCl and NaOH solution streams at different concentrations were injected to the extruder at the mixing zone. The 15, 10, and 5% (v/v) HCl solutions were used to create the pH of 2.89, 3.53, and 4.44, respectively. In addition, the 0.83 and 1.67% (w/v) NaOH solution streams were injected to the extruder to create the pH of 7.01 and 8.16, respectively. These were compared with WPC-based blend extruded with deionized water stream only (non-pH-adjusted). A pilot scale supercritical fluid system was used for injecting SC-$CO_2$ into the protein polymer melts at L/D of 24 through four injection valves located around the extruder barrel. The die pressure was maintained higher than the pressure inside the barrel for continuous SC-$CO_2$ flow into the protein polymer melt, at the desired rate (1% dry basis) and pressure (10-15 MPa). Product temperatures were monitored by a thermocouple at the end of the extruder. The extrudates were collected, dried at room temperature overnight or until their moisture contents were between 5 and 7%, and stored at room temperature in sealed containers.

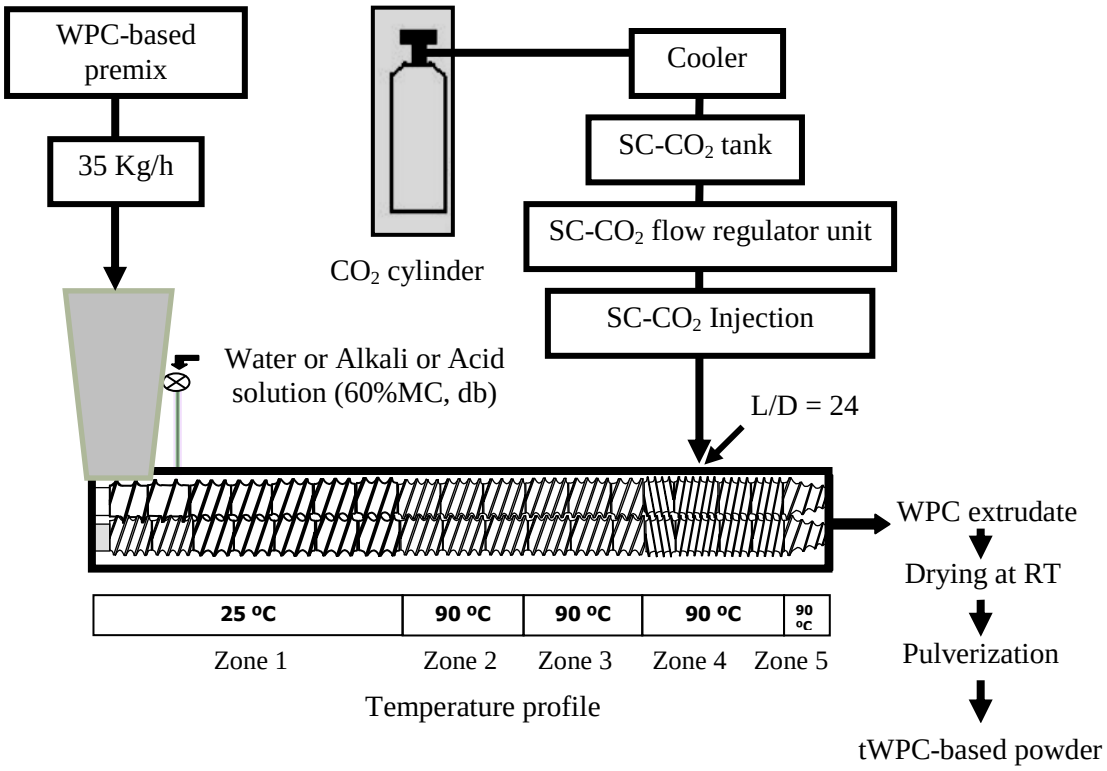


Figure 2.1. Schematic of WPC-based texturization by supercritical fluid extrusion (SCFX), screw configuration, and temperature zones.

### 2.3.3. tWPC-based powder preparation

Dried tWPC-based extrudates were grounded into powders using the mill machine (model 8000M-115, SPEX CertiPrep, LLC, Metuchen, NJ, USA) to reduce the particle size to less than or equal to 125 μm. All samples were then stored at room temperature in air tight containers until analyzed.

### 2.3.4. Rheological characterizations

#### 2.3.4.1. Viscosity measurement and flow behavior by shear rate ramp test

The tWPC and unprocessed WPC-based (control) powders were reconstituted at 20% (w/w) concentration in deionized water and gently stirred for 2 h or until dissolution was completed, and then stored overnight at 4 ºC prior to testing. This was done to ensure that dispersions were in the fully recovered state. The parallel plate geometry with 50 mm plate diameter was utilized for steady shear rate ramp test. The sample was loaded into the rheometer (ARES strain-controlled rheometer, TA Instruments, New Castle, DE, USA) equipped with a Peltier temperature controlling system, and then the top plate was slowly lowered until the final sample thickness of 1 mm was achieved. A thin layer of mineral oil was applied to the exposed sample edges to prevent the moisture loss. All experiments were conducted at 25 ºC. Shear rate was ramped from 1 to 100 $s^{-1}$. Shear stress (τ), shear rate ($\dot{\gamma}$), and apparent viscosity ($\eta_a$) were recorded by TA Orchestrator software. The corrected flow curves were fitted using the power law (Eq.1) and Herschel-Bulkley model (Eq. 2). The flow behavior index (n), consistency coefficient (k), and yield stress ($\tau_{0HB}$) were reported.

$$\tau = k\dot{\gamma}^n \tag{1}$$

$$\eta_a = \frac{\tau_{OHB}}{\dot{\gamma}} + k\dot{\gamma}^{n-1} \tag{2}$$

To investigate the effect of protein concentration, tWPC and unprocessed WPC dispersions were prepared at concentrations of 10%, 12.5%, 15%, 17.5% and 20% (w/w). These samples were allowed to hydrate overnight, and subjected to the same shear rate ramp test at 25 ºC. All determinations were done in triplicates.

### 2.3.4.2. Viscoelastic properties by small-amplitude oscillatory (dynamic) tests

#### 1) Frequency sweep test

The tWPC and unprocessed WPC-based (control) powders were reconstituted (30%, w/w) in deionized water and gently stirred for 2 h at ambient temperature and allowed to hydrate overnight at 4 ºC. Viscoelastic properties of all samples were monitored using the rheometer (ARES strain-controlled rheometer, TA Instruments, New Castle, DE, USA) equipped with a Peltier temperature controlling system, and utilizing a parallel plate geometry (25 mm plate diameter). The sample was loaded into the rheometer, and then the top plate was slowly lowered until the final sample thickness of 2 mm was achieved. A thin layer of mineral oil was applied to the exposed sample edges to prevent moisture loss. The frequency was oscillated from 0.1 to 100 rad/s at 25 ºC. All measurements were performed within the identified linear viscoelastic region and made at 1% strain. The storage modulus ($G'$), loss modulus ($G''$), complex viscosity ($\eta^*$), and loss angle tangent (tan $\delta$) were then recorded by TA Orchestrator software. All determinations were done in triplicates

#### 2) Temperature sweep test

The thermal stability of selected 20% (w/w) tWPC and unprocessed WPC dispersions were monitored using the same rheometer and procedure with 25 mm diameter parallel plate geometry, and 2 mm sample thickness. The temperature was ramped from 25 ºC to 85 ºC at 2 ºC/min heating rate and at a constant frequency rate

of 1 rad/s and 1% strain. The storage modulus ($G'$), loss modulus ($G''$), complex viscosity ($\eta^*$), and loss angle tangent (tan $\delta$) were then recorded by TA Orchestrator software. All determinations were done in triplicates

### 2.3.5. Water holding capacity (WHC) of tWPC powders

The selected tWPC powders were hydrated (15%, w/w) in deionized water for 3 h and centrifuged at 3,500 RPM for 30 min at 25 ˚C. After centrifugation, the supernatant was removed and the remaining pellet was weighed. The amount of water held per gram of protein powder was calculated as the WHC. All determinations were done in triplicates

### 2.3.6. Statistical Analysis

Statistical analysis was done using MINITAB® release 14 statistical software (State College, PA, USA). Significant differences ($p$<0.05) were determined by analysis of variance using the general linear models and least square means procedure.

## 2.4. Results and discussion

### 2.4.1. General observation on tWPC production

The 6% (w/w) pre-gelatinized corn starch was added to the feed formulation for the extrusion process in order to facilitate the manufacturing of tWPC extrudates. The preliminary studies revealed that the extrusion process of 100% WPC powder was difficult to achieve. The pre-gelatinized corn starch has been used as a binder to hold protein matrices because of their ability to form hydrogen bonds in the extruded products (Amaya-Llano, Morales Hernández, Castaño Tostado, & Martínez-Bustos, 2007). Considerably, in this process the pre-gelatinized corn starch acted as an inactive filler in the tWPC extrudate formation. According to Aguilera and Rojas

(1996) the rheological properties of heat-induced WP gels were significantly influenced when 10-20% (w/w) of WP in the system was substituted by corn starch. However, a treatment with WPC only was preliminarily investigated in order to assess the effects of starch on the final gel characteristics of the extruded WPC samples. It showed that the addition of 6% (w/w) pre-gelatinization corn starch to the feed formulation did not have a significant effect on the rheological characteristics of final tWPC products (data not shown) due to the apparently low starch content (0.6-1.8%, w/w) in the studied WP dispersions. Therefore, the interaction between starch and protein would be insignificant, given that the structure of the extrudates was dominated by protein-protein interactions during extrusion. The main factor in this study that could influence and dominate the structural changes in WP was extrusion pH. Hudson and Daubert (2002), and Shim and Mulvaney (2001) indicated that changes in WP structure were significantly dependent on pH while starch was relatively insensitive to pH changes.

The extrusion process was more severe in alkaline than in acidic conditions. Onwulata et al. (2006) reported similar observations during extrusion of WPI under alkaline conditions. A stronger alkaline treatment produced the WP extrudate with a dry rough surface. Based on the visual observation, alkaline conditions increased the dark yellow color and the toughness of the extrudates. The acidic conditions, on the other hand, resulted in transparent and light yellow color extrudates with a smooth surface, which fractured more ease after the drying process.

Injection of 1% (db) SC-$CO_2$ to the pH-treated tWPC slightly reduced the pH of the final products. The incorporation of SC-$CO_2$ into protein polymer melts in the extruder was achieved by injecting it above its critical conditions ($T_c$=31 ºC and $P_c$=7.38 MPa) (Chen, 2005). In supercritical phase, SC-$CO_2$ is readily dissolved into the water phase in the protein polymer melts, and forms carbonic acid providing

additional acidity to final tWPC products. Injection of SC-$CO_2$ to the pH-treated WPC melts produced a lighter product color visually apparent in most extrudate samples.

### 2.4.2. Apparent viscosity

The variation in the apparent viscosity of dispersions containing 20% (w/w) tWPC powders produced at different pH compared with the unprocessed WPC blend is illustrated in Figure 2.2a. The viscosity of most tWPC samples was shear rate dependence, except for the tWPC produced at pH 4.44 which appeared to be Newtonian similar to that of the unprocessed WPC. Data revealed in Table 2.1 indicated that the acid-treated-tWPC samples had apparent viscosities at 25 $s^{-1}$ shear rate ($\eta_{25}$) ranging from 0.026 to 0.988 Pa s while the alkali-treated tWPC samples had the $\eta_{25}$ of 0.292 to 0.444 Pa s. The non-pH treated tWPC had the average $\eta_{25}$ of 0.052 Pa s while the unprocessed WPC blend showed the lowest $\eta_{25}$ of 0.008 Pa s. The enhanced apparent viscosity was shown in most of the pH-treated tWPC samples. This indicated an increase in unfolding and denaturation of globular proteins when the pH of WPC was altered. In general, unfolding of globular proteins is frequently accompanied by an increase in the hydrodynamic radii of protein molecules and greater molecular entanglements which contribute to an increase in the apparent viscosity of WP solutions (Rattray & Jelen, 1995). Our finding suggests that adjusting acidity ($H^+$) and alkalinity ($OH^-$) of WPs while heating and shearing during extrusion could induce the conformational structure alteration of proteins since their net charges have been manipulated. According to Harding (1998), proteins are all polyelectrolytes and possess electrostatic charge which can affect the viscosity. This electrostatic contribution is strongly dependent on the pH and the ionic strength of solution. As pH was adjusted away from the isoelectric point (pH 5.0-5.3), the viscosity of WP solutions significantly increased (Rector, 1992).

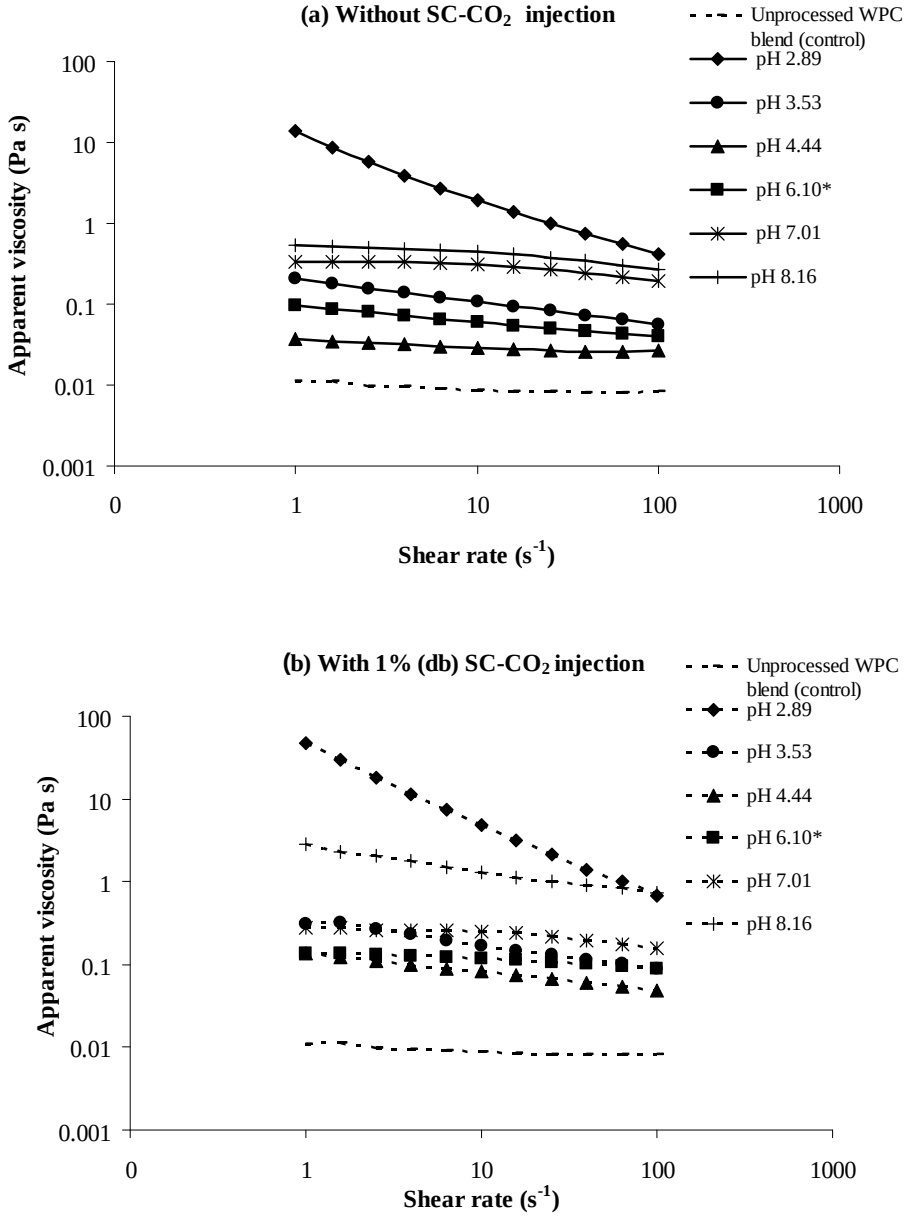


Figure 2.2. Variation of apparent viscosity with shear rate for dispersions containing 20% (w/w) tWPC powders extruded at different pH; (a) without SC-$CO_2$, and (b) with 1% (db) SC-$CO_2$ injection and unprocessed WPC blend powder. Data are averages for 3 tests. The maximum error in apparent viscosity data was 5%. *tWPC powder extruded with water only (non-pH-adjusted).

Table 2.1. Rheological parameters[a] of models describing flow behaviors of dispersions containing 20% (w/w) tWPC and unprocessed WPC-based powders.

| Extrusion pH | $\eta_{25}$ [Pa s] | Power law model | | | Herschel-Bulkley model | | | |
|---|---|---|---|---|---|---|---|---|
| | | $k$ [Pa s$^n$] | $n$ | $R^2$ | $\tau_{0HB}$ [Pa] | $k$ [Pa s$^n$] | $n$ | $R^2$ |
| Unprocessed WPC blend (control) | 0.008 | 0.011 | 0.928 | 0.999 | 0.000 | 0.009 | 0.989 | 0.999 |
| Without SC-$CO_2$ | | | | | | | | |
| 2.89 | 0.988 | 11.731 | 0.250 | 0.953 | 10.820 | 2.290 | 0.560 | 0.999 |
| 3.53 | 0.083 | 0.203 | 0.720 | 1.000 | 0.010 | 0.196 | 0.733 | 1.000 |
| 4.44 | 0.026 | 0.035 | 0.922 | 0.999 | 0.010 | 0.028 | 0.969 | 0.999 |
| 6.10[b] | 0.052 | 0.095 | 0.807 | 0.999 | 0.022 | 0.082 | 0.855 | 0.999 |
| 7.01 | 0.292 | 0.370 | 0.883 | 0.997 | 0.400 | 0.638 | 0.740 | 0.999 |
| 8.16 | 0.442 | 0.579 | 0.855 | 0.998 | 0.800 | 1.022 | 0.716 | 0.999 |
| With 1% (db) SC-$CO_2$ | | | | | | | | |
| 2.89 | 2.063 | 43.028 | 0.078 | 0.771 | 42.480 | 1.500 | 0.560 | 0.985 |
| 3.53 | 0.122 | 0.338 | 0.711 | 0.999 | 0.118 | 0.246 | 0.770 | 0.999 |
| 4.44 | 0.068 | 0.136 | 0.778 | 1.000 | 0.005 | 0.133 | 0.789 | 0.999 |
| 6.10[b] | 0.025 | 0.144 | 0.906 | 0.999 | 0.001 | 0.026 | 0.990 | 0.999 |
| 7.01 | 0.249 | 0.303 | 0.884 | 0.998 | 0.400 | 0.538 | 0.740 | 0.999 |
| 8.16 | 1.031 | 2.650 | 0.710 | 0.998 | 1.618 | 2.271 | 0.750 | 0.998 |

[a]Mean of triplicates; $\eta_{25}$—apparent viscosity at 25 s$^{-1}$ shear rate; $k$—consistency coefficient; $n$—flow behavior index; $\tau_{0HB}$—yield stress.
[b]tWPC powder extruded with water only (non-pH-adjusted).

The tWPC produced at extremely acidic condition of pH 2.89 yielded the highest apparent viscosity while the products at pH 3.53 and 4.44 evidently exhibited much lower apparent viscosities at any given shear rate. At pH 2.89, the apparent viscosity of WPC has been ultimately improved by approximately 124 times ($\eta_{25}$ = 0.988 Pa s) compared to the unprocessed WPC sample ($\eta_{25}$ = 0.008 Pa s) (Table 2.1). It was also observed that at 20% (w/w) WP dispersion, tWPC produced at pH 2.89 yielded a highly homogeneous, smooth, and viscous dispersion texture. It is interesting to note that an increase in apparent viscosity of highly acid-treated tWPC is the evitable outcome of acid-induced protein denaturation (Rattray & Jelen, 1995). The denaturation of α-lacalbumin (α-La) and bovine serum albumin (BSA) at pH 3.5 or lower were documented (Bernal & Jelen, 1984; Boye, Kalab, Alli, & Ma, 2000). However, β-lactoglobulin (β-Lg), the most abundant protein in whey (~50% of total WP), is more rigid and resistant to extensive denaturation at acid pH (pH < 3.5) (de Wit & Klarenbeek, 1984; Jelen & Buchheim, 1984; Monahan, German, & Kinsella, 1995; Taulier & Chalikian, 2001). As described earlier, heating and shearing in the extruder led to protein unfolding, thus rendering the proteins more susceptible to further denaturation by acid treatment. Perhaps by thermal and mechanical energy provided by extrusion process, β-Lg could be more denatured and contributed to high apparent viscosity or gelation at low pH. Accordingly, Rattray and Jelen (1995) reported that thermal treatment of acidic WPC solutions (11% and 20%, w/v, protein) at pH values < 3.5 caused relatively strong gels due to protein denaturation and aggregation. A similar observation was also reported by Singer, Yamamoto, and Latella (1988). The authors demonstrated that a highly viscous, semi-solid product with lipid-like texture could be produced by simultaneously heating and shearing of liquid WPC process at acid pH.

It is evident that increasing pH of WPC to 8.16 resulted in a moderate increase in apparent viscosity (Table 2.1). As reported by Monahan et al. (1995), at alkali pH, proteins attain an overall negative charge groups. If enough negative charge is present, adjacent portions of the protein will start to repel each other due to the high charge density. When this occurs, the protein begins to unfold and the molecules' radius of gyration should expand and result in an increased viscosity. Consequently, the exposure of sulfhydryl (SH-) group and thiol-disulfide interchange are expected to occur which leads to a higher rate of aggregation and denaturation (Watanabe & Klostermeyer, 1976). Therefore, extruding WPC in highly alkaline conditions could produce larger aggregates and/or high molecular weight denatured proteins. The tWPC produced at extremely alkaline condition (pH 8.16) exhibited a coarse and gritty dispersion texture due to the higher extent of insoluble large aggregated proteins contained in the sample. This was also observed in our SDS-PAGE electrophoresis studies (data not shown). However, WPs with a higher degree of aggregation were found to have increased viscosity due to the aggregates greater effective volume which could entrap more water than the individual WP molecules (Firebaugh, 2004). These findings are similar to those of texturized soy protein studied by Dahl and Villota (1991) and Fleming, Sosulski, Kilara, and Humbert (1974). Their results indicated that the viscosity of soy proteins increased markedly after proteins were modified by the alkaline treatment.

The combined effects of SC-$CO_2$ and pH treatment on the apparent viscosity of tWPC dispersions are illustrated in Figure 2.2b. It is clearly shown that injecting 1% (db) SC-$CO_2$ into pH-adjusted WP polymer melts generally improved the apparent viscosity of final tWPC products at comparable shear rate. As shown in Table 2.1, incorporation of SC-$CO_2$ noticeably improved the viscosity of tWPC samples extruded at pH 2.89, 3.53, 4.44 and 8.16. Considerably, the effect of SC-$CO_2$ addition on the

viscosity of tWPC samples became more pronounce when WPC blend was extruded at extremely acidic (pH 2.89) or alkaline (pH 8.16) conditions. At extreme acid condition, the apparent viscosity ($\eta_{25}$) of tWPC dispersion significantly ($p < 0.05$) increased from 0.988 Pa s to 2.063 Pa s with SC-$CO_2$ addition. For extreme alkali condition, the $\eta_{25}$ of tWPC dispersion significantly ($p < 0.05$) increased from 0.442 Pa s to 1.031 Pa s with SC-$CO_2$ addition. Overall, incorporation of 1% (db) SC-$CO_2$ contributed approximately 258 and 129 times higher viscosity in tWPC produced at pH 2.89 and 8.16, respectively, compared to the unprocessed WPC sample ($\eta_{25}$ = 0.008 Pa s). Results revealed that tWPC produced under extremely acidic condition of pH 2.89 with SC-$CO_2$ treatment yielded the maximum apparent viscosity at equal shear rate. Combination of highly acid environment and SC-$CO_2$ addition by SCFX process possibly influenced hydrophobic interactions versus non-covalent linkages, such as intermolecular hydrogen bonding and electrostatic interactions. These interactions are found to be important in hydrocolloids to function as thickening agents in some food products such as salad dressing and sauces (Lang & Rha, 1981).

### 2.4.3. Flow behavior

The decreasing viscosity when shear rate is increasing observed in most tWPC samples implies the pseudoplastic flow behavior or shear thinning behavior which is commonly seen in food thickeners (Daubert, Resch, & Foegeding, 2004). To study their flow behaviors, shear stress-shear rate data of dispersions (20% protein concentration, w/w) of pH-treated tWPC with and without SC-$CO_2$ injection (Figure 2.3a and 2.3b) and the unprocessed WPC were fitted to two different rheological models, the power law and Herschel-Bulkley. The power law model does not take account of yield stress. On the other hand, the Herschel-Bulkley model contains yield stress ($\tau_{0HB}$) which is a very important rheological parameter and has been often used

to explain the flow behavior of pseudoplastic material properties (Curran, Hayes, Afacan, Williams, & Tanguy, 2002; Hudson & Daubert, 2002). The yield stress ($\tau_{0HB}$), consistency coefficient ($k$), and flow behavior index ($n$) of tWPC dispersions compared to the unprocessed WPC were summarized in Table 2.1. The coefficients of determination ($R^2$) for each sample were always greater than 0.95, supporting the validity of the selected models. Although the flow behaviors of tWPC dispersions were represented well by both models, the Herschel-Bulkley model exhibited a better fit in most samples regarding the higher $R^2$ values. This occurred because most tWPC samples displayed the yield stress values as shown in Table 2.1.

The flow behavior index ($n$) values were generally greater for the Herschel-Bulkley model than for the power law model. This was probably due to the different fit of the two models to the experimental data and to the fact that the power law model disregards yield stress. However, the $n$ values from both models were less than 1.0, implying the pseudoplasticity of most tWPC samples. Nonetheless, the tWPC powder produced at pH 4.44 and the unprocessed WPC exhibited Newtonian behavior confirmed by their n values which approached 1.0. Results depicted in Table 2.1 show that the n values from the Herschel-Bulkley model for pH-treated tWPC with injected SC-$CO_2$ is in the range of 0.56 to 0.78. This indicates the shear-thinning or pseudoplastic nature of particulate aggregate or weak gel type of flow (Rector, 1992). The tWPC treated with extreme acid (pH 2.89) and SC-$CO_2$ showed the greatest values of shear stresses in a given range of shear rates with markedly highest yield stress (Figure 2.3b). It also had the highest consistency coefficient and lowest flow behavior index based on the power law model (Table 2.1). This result is also confirmed by the steepest viscosity rise at low shear rate presented in Figure 2.2b. It can be attributed to the breakdown of the inner structure of fluid which is formed through physical interactions between molecules (Gerhards & Schubert, 1993).

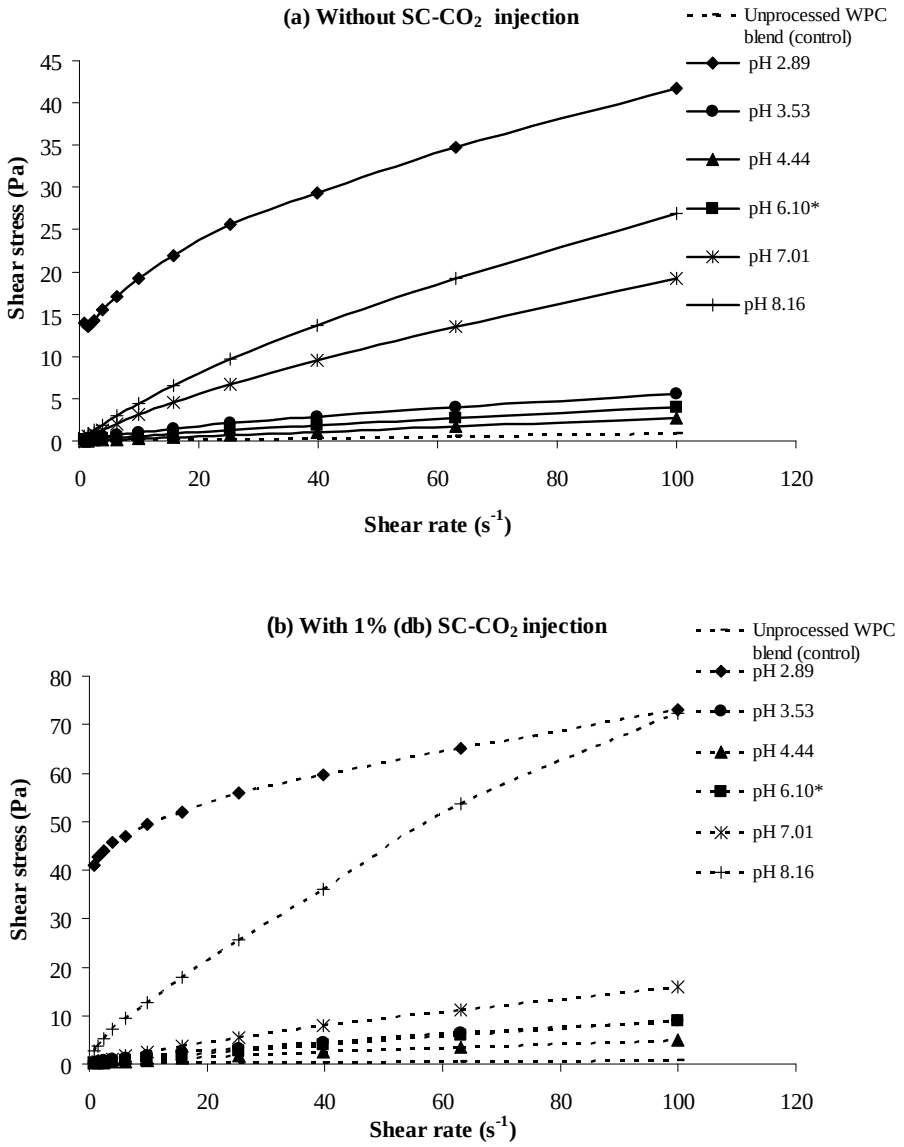


Figure 2.3. Shear stress-shear rate relationship of dispersions containing 20% (w/w) tWPC powders extruded at different pH; (a) without SC-$CO_2$, and (b) with 1% (db) SC-$CO_2$ injection and unprocessed WPC blend powder. Data are averages for 3 tests. The maximum error in shear stress-shear rate data was 5%. *tWPC powder extruded with water only (non-pH-adjusted).

As the shear rate increases, those forces weaken and the molecules orient themselves along the flow lines, which causes a drop in viscosity. The higher yield stress represents the stronger gel network in which higher stress is required to break a gel and initiate flow. Hudson and Daubert (2002) reported that the yield stress appeared in derivatized WPI was due to hydrophobic interactions versus non-covalent linkages. Such result would suggest that SCFX process rendered WPC into a product with thickening characteristics.

### 2.4.4. Influence of protein concentration

The effect of protein concentrations (10, 12.5, 15, 17.5, and 20%, w/w) on the apparent viscosity at 61.15 $s^{-1}$ shear rate ($\eta_{61.15}$) of tWPC samples with and without SC-$CO_2$ treatment is illustrated in Figure 2.4a and 2.4b. Results revealed that all tWPC samples exhibited the ability to impart a wide range of viscosities by varying protein concentrations. It clearly shows that the apparent viscosity of most samples increased with the increase in protein concentration. With 1% (db) SC-$CO_2$ injection, the greater apparent viscosity of tWPC samples was observed in all concentrations. Drastic increase in apparent viscosity at higher protein concentrations was observed in tWPC samples produced at pH 2.89 and 8.16 with SC-$CO_2$ treatment. Notably, the tWPC produced at pH 2.89 with SC-$CO_2$ noticeably yielded the highest apparent viscosity at equal concentration, and its apparent viscosity increased by 22 times when the concentration of protein increased from 10 to 20% (w/w). On the other hand, the protein concentration did not largely influence the apparent viscosity of unprocessed WPC sample. Clark (1998) reported that the native WP will not usually achieve a high viscosity, even at moderately high concentrations, because of its folded globular shape. However, when WPs were more denatured, the increase in viscosity became more pronounced as generally observed in several tWPC samples.

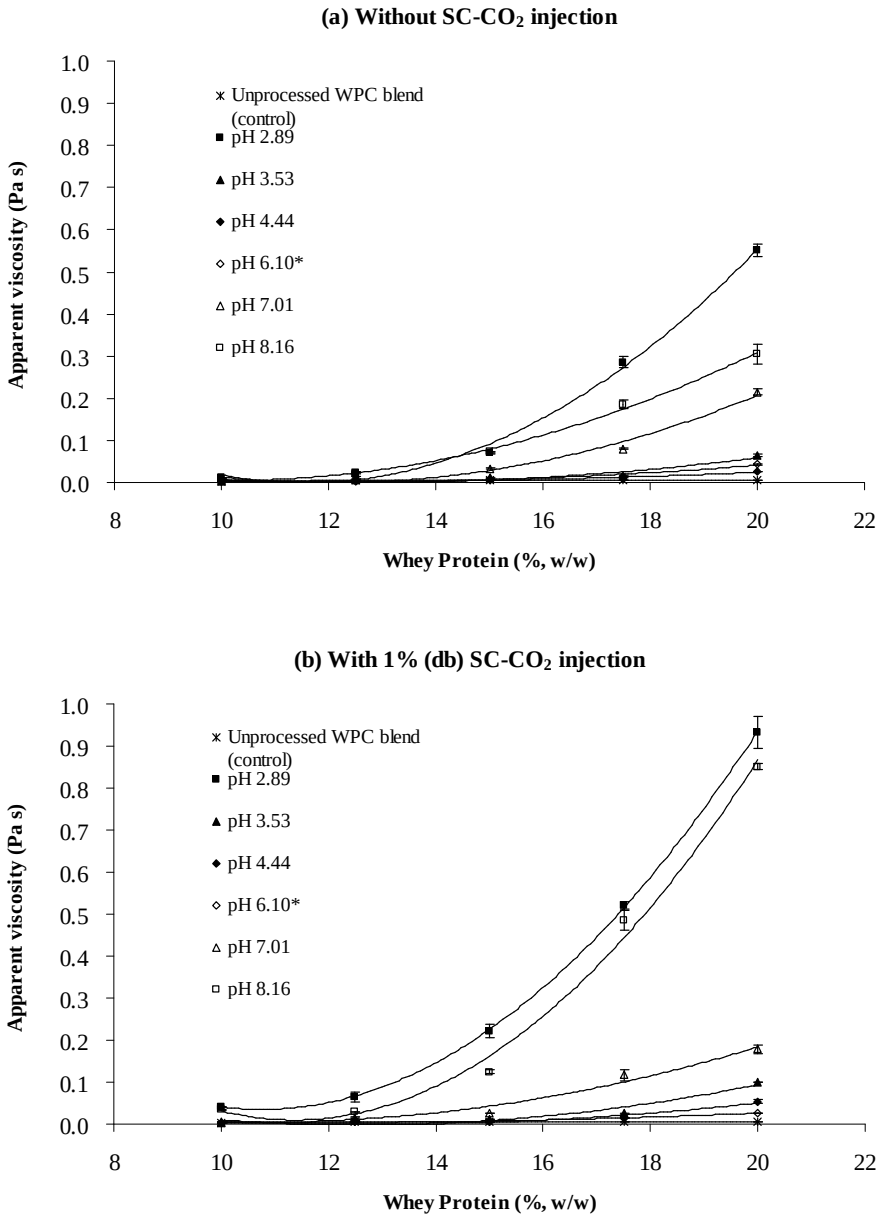


Figure 2.4. Variation of apparent viscosity (at 61.15 $s^{-1}$ shear rate) with WP concentrations for dispersions containing tWPC powders extruded at different pH; (a) without SC-$CO_2$, and (b) with 1% (db) SC-$CO_2$ injection and unprocessed WPC blend powder. Error bars represent standard deviation from the mean of 3 trials. *tWPC powder extruded with water only (non-pH-adjusted).

At higher protein concentrations, it was likely that denatured proteins were packed closely together, promoting protein-protein interactions, greater protein molecular entanglements, and polymerization, all of which contributed to the viscosity increases (Rector, 1992). In addition, swelling caused by hydrogen bonds between amino acid groups and water, resulting in an increase in the molecular radii of protein molecules could considerably be involved in the viscosity increase as well (Clark, 1998; Rattray & Jelen, 1995; Schmidt, Packard, & Morris, 1984). Thus, stabilizing and strengthening characteristics shown in of tWPC dispersions were more pronounced as the amount of denatured proteins increased.

### 2.4.5. Viscoelastic properties

In this study, the dynamic mechanical testing approach was used to measure mechanical changes in linear viscoelastic behavior of tWPC samples containing 30% (w/w) WP. Unlike the viscosity measurement, this small strain measurement is believed to leave microstructure intact and thus be able to characterize viscoelastic properties of the original fluid structure (Gunasekaran & Ak, 2000). A viscoelastic network indicates the elastic and viscous behavior of the sample over a range of frequencies. The storage modulus ($G'$) represents a measure of elastic response of the material whilst the loss modulus ($G''$) is a measure of the viscous response.

The unprocessed WPC data have not been included in the viscoelastic results due to its very poor consistency which prohibited measurement of its dynamic properties in the linear viscoelastic range. It did not show viscoelastic behavior as observed from its very low viscosity ($G'' \gg G'$) and it was impossible to obtain the plateau modulus. Results illustrated in Figure 2.5a (without SC-$CO_2$) and 2.5b (with SC-$CO_2$) revealed that $G'$ of all tWPC samples increased as the frequency increased from 0.1 to 100 rad/s. The higher $G'$ than $G''$ over a range of frequency has been found

in all tWPC samples (data not shown for $G''$). This testifies to the dominance of elastic properties over viscous ones. The similar characteristics have been also reported for starch and protein gels and mixed starch/WP gels (Beveridge & Timbers, 1985; Carvalho, Onwulata, & Tomasula, 2007; El-Garawany & Abd El Salam, 2005). The authors described that this behavior could be attributed to the cross-linkage formations by disulfide bonds and hydrophobic interactions in the gel structures. The lowest pH (2.89) and highest pH (8.16) tWPC samples had excessively large $G'$, while pH 6.10 and 7.01 samples had the moderate $G'$, and pH 3.53 and 4.44 samples showed the lowest $G'$ over a range of frequencies (Figure 2.5a).

The elastic modulus ($G'$), loss modulus ($G''$), tan δ, and complex viscosity ($\eta^*$) of tWPC and unprocessed WPC samples were compared as shown in Table 2.2. The frequency of oscillation chosen for comparison was 1 rad/s, which represents a timescale sufficiently short that even physical gel cross-links are effectively permanent. It was likely that by adjusting pH, the viscoelastic properties of WPC blend were manipulated, especially at highly alkaline and acidic environments. Extruding WPC blend at extremely acidic concentration (pH 2.89) significantly ($p < 0.05$) yielded the highest $G'$ (4798.43 Pa), $G''$ (975.41 Pa), and $\eta^*$ (4896.62 Pa s), followed by the highly alkaline treatment of pH 8.16 ($G'$=570.59 Pa, $G''$=111.08 Pa, and $\eta^*$=581.33 Pa s). The moderate $G'$, $G''$, and $\eta^*$ were observed in pH 6.10 and 7.01 samples, while the pH 3.53 and 4.44 samples possessed the lowest $G'$, $G''$, and $\eta^*$ values.

SCFX process with 1% (db) SC-$CO_2$ injection is shown to improve the viscoelastic properties of most pH-treated tWPC samples. The most pronounced changes in viscoelastic properties when the SC-$CO_2$ was incorporated were found in alkali-treated tWPC at pH 7.01, where $G'$, $G''$, and $\eta^*$ increased by approximately 8, 6, and 8 times, respectively (Table 2.2).

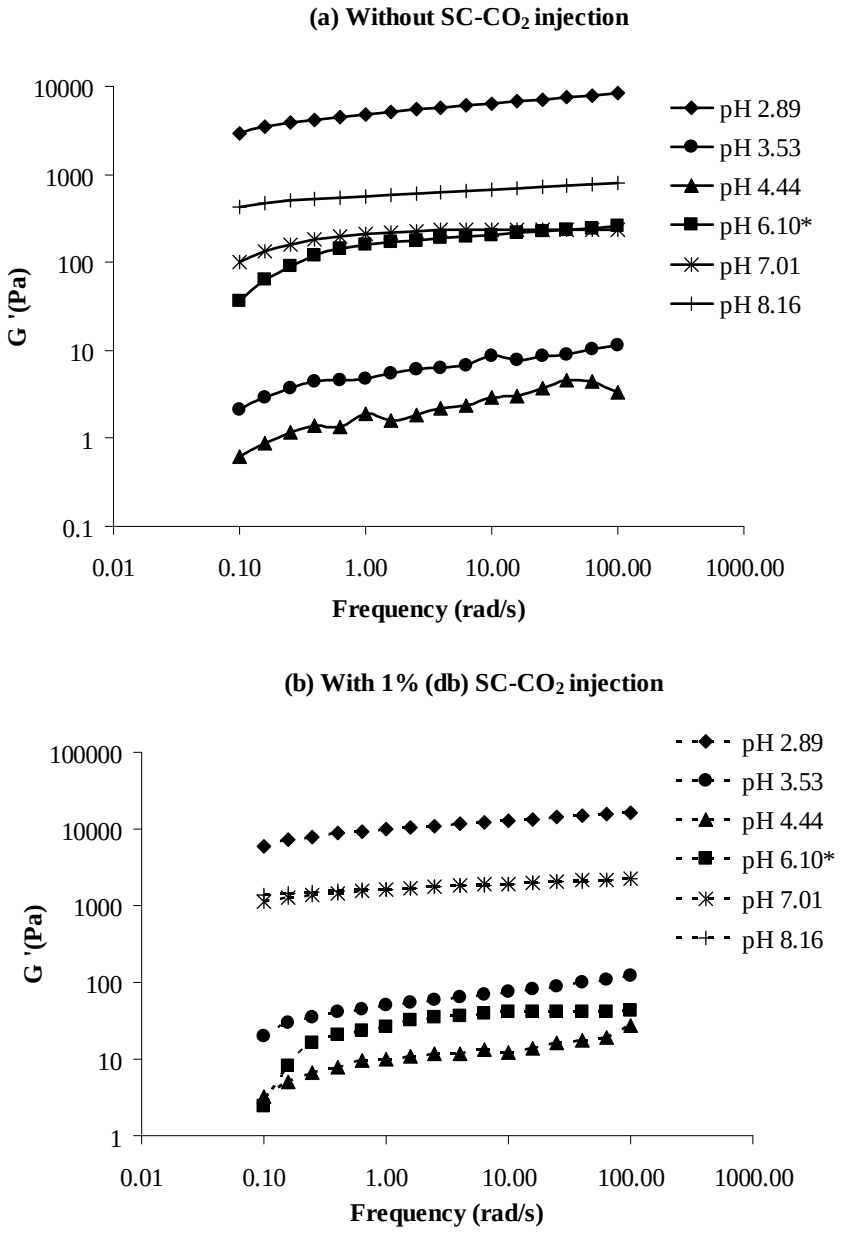


Figure 2.5. Variation of storage (elastic) modulus (*G′*) with frequency for dispersions containing 30% (w/w) tWPC powders extruded at different pH; (a) without SC-$CO_2$, and (b) with 1% (db) SC-$CO_2$ injection. Data are averages for 3 tests. The maximum error in *G′* data was 5%. *tWPC powder extruded with water only (non-pH-adjusted).

Table 2.2. Storage modulus ($G'$), loss modulus($G''$), tan $\delta$, and complex viscosity ($\eta^*$) at the frequency of 1 rad/s of dispersions containing 30% (w/w) tWPC and unprocessed WPC-based powders[a].

| Extrusion pH | $G'$ [Pa] | $G''$ [Pa] | tan $\delta$ | $\eta^*$ [Pa s] |
|---|---|---|---|---|
| Unprocessed WPC blend (control) | 0.036[a] | 0.075[a] | 2.083[a] | 0.083[a] |
| <u>Without SC-$CO_2$</u> | | | | |
| 2.89 | 4798.435[e] | 975.410[e] | 0.203[g] | 4896.622[e] |
| 3.53 | 4.327[a] | 2.363[a] | 0.546[b] | 5.250[a] |
| 4.44 | 1.895[a] | 1.044[a] | 0.550[b] | 2.168[a] |
| 6.10[b] | 156.853[b] | 52.484[b] | 0.335[e] | 165.269[b] |
| 7.01 | 210.755[b] | 64.371[b] | 0.305[ef] | 220.384[b] |
| 8.16 | 570.591[c] | 111.081[c] | 0.195[h] | 581.333[c] |
| <u>With 1% (db) SC-$CO_2$</u> | | | | |
| 2.89 | 9885.870[f] | 1805.123[f] | 0.183[gh] | 10049.755[f] |
| 3.53 | 50.169[a] | 16.576[a] | 0.330[e] | 52.828[a] |
| 4.44 | 9.966[a] | 4.695[a] | 0.479[c] | 11.017[a] |
| 6.10[b] | 26.663[a] | 10.538[a] | 0.395[d] | 28.679[a] |
| 7.01 | 1617.065[d] | 358.865[d] | 0.221[f] | 1656.413[d] |
| 8.16 | 1651.812[d] | 314.813[d] | 0.196[g] | 1681.645[d] |

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

[b]tWPC powder extruded with water only (non-pH-adjusted).

However, injection of SC-$CO_2$ to the extreme acid-treated tWPC significantly ($p < 0.05$) yielded the highest $G'$ (9885.87 Pa), $G''$ (1805.12 Pa), and $\eta^*$ (10049.75 Pa s), compared to the unprocessed WPC ($G'$= 0.036 Pa, $G''$ = 0.075 Pa, and $\eta^*$= 0.083 Pa s). It is clearly shown that extruding WPC blend at pH 2.89 with SC-$CO_2$ injection ultimately increased its $G'$, $G''$, and $\eta^*$ by approximately 274,000, 24,000, and 120,000 times, respectively, compared to the unprocessed WPC.

The relative 'strength' of gels could be interpreted in terms of tan $\delta$ ($G''/G'$), measuring energy loss compared to energy stored in cyclic deformation. It was indicated that gels with tan $\delta > 0.1$ had a paste-like quality (weak gel), while gels with tan $\delta < 0.1$ were firm, self-standing (true) gels (Clark, 1998; Shim & Mulvaney, 2001). In this study, tWPC samples showed a broader range of tan $\delta$ compared at the same frequency of 1 rad/s (0.183 to 0.550) which is greater than 0.1, indicating a weak gel or paste-like structure. A relative comparison of tan $\delta$ was shown over the entire range of frequencies for pH-treated tWPC without SC-$CO_2$ (Figure 2.6a) and with 1% (db) SC-$CO_2$ injection (Figure 2.6b). With SC-$CO_2$ addition, most tWPC samples showed a lower extent of tan $\delta$ over a range of frequencies, indicating the more solid gel behaviors, except for the non-pH adjusted tWPC (pH 6.10) sample. The almost linear relationship between tan $\delta$ and the applied frequencies of those samples also indicates the stronger gel networks and more solid-like behaviors. Figure 2.6b and Table 2.2 reveal that the tWPC produced at pH 2.89 with SC-$CO_2$ injection significantly ($p < 0.05$) yielded the strongest gel network indicated by the lowest tan $\delta$ (0.183).

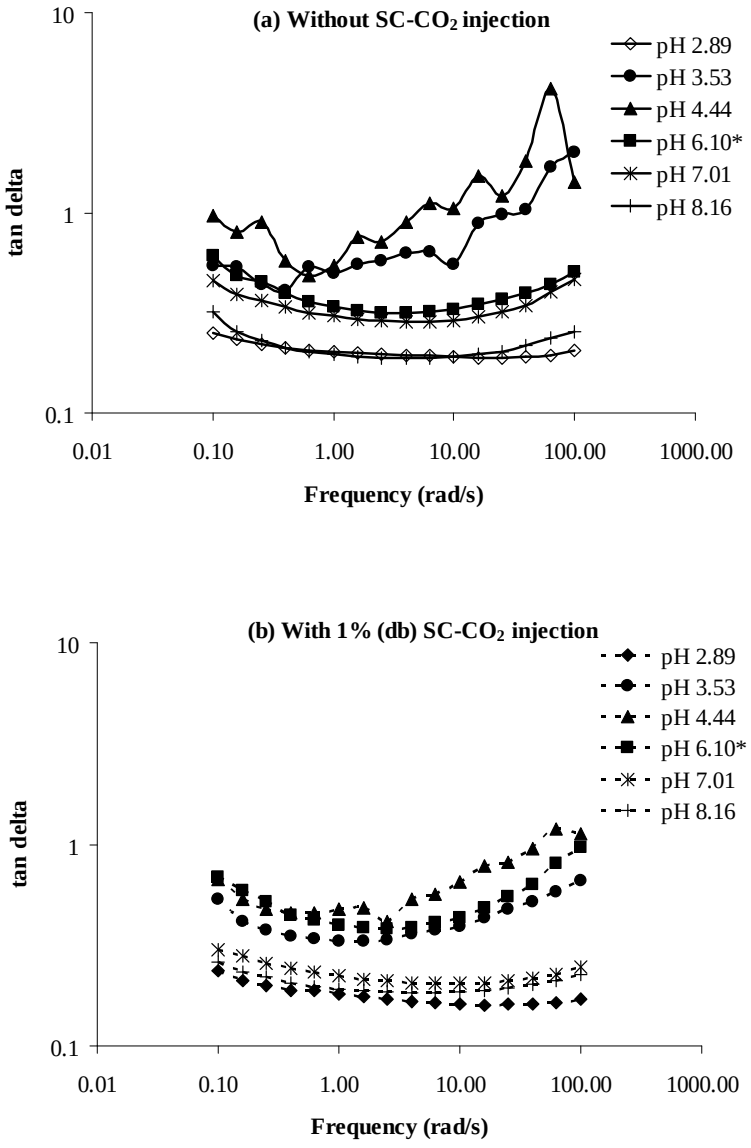


Figure 2.6. Variation of tan delta (tan $\delta$) with frequency for dispersions containing 30% (w/w) tWPC powders extruded at different pH; (a) without SC-$CO_2$, and (b) with 1% (db) SC-$CO_2$ injection. Data are averages for 3 tests. The maximum error in tan $\delta$ data was 5%. *tWPC powder extruded with water only (non-pH-adjusted).

It was reported that the absolute difference between $G'$ and $G''$ over a range of frequencies and the degree of frequency independency of moduli demonstrated the typical characteristics of gels (Clark, 1998; Rodd, Davis, Dunstan, Forrest, & Boger, 2000; Shim & Mulvaney, 2001). Table 2.3 shows the frequency dependence of the moduli ($G'$ and $G''$) analyzed quantitatively by fitting simple power law relationships;

$$G' \; a \; \omega^{p} \tag{3}$$

$$G'' \; a \; \omega^{q} \tag{4}$$

where $\omega$ is the frequency of oscillation and $p$ and $q$ are the storage and loss moduli power law indices, respectively. The values of $p$ and $q$, determined in this study, showed a slightly wide range of 0.069 to 0.279 for $p$, and 0.052 to 0.445 for $q$. The results shown in Table 2.3 confirm that accompanying SC-$CO_2$ with pH alteration using SCFX process produced a stronger gel network in most tWPC samples indicated by lower extent of frequency dependence of moduli (lower $p$ and $q$ values). Results indicated that the tWPC produced at pH 2.89, 7.01 and 8.16 with SC-$CO_2$ had a noticeably lowest frequency dependence of $G'$ and $G''$, implying the stronger internal structure compared to the rest of the samples. However, tWPC samples did not resemble those of solid gel characteristics regarding their frequency dependence of mechanical spectra ($G'$, $G''$, and tan $\delta$) as previously presented. In addition, the differences between $G'$ and $G''$ values of all samples were less than one order of magnitude, indicating a weak gel entanglement network (Clark & Ross-Murphy, 1987). Among the samples studied, tWPC produced at pH 2.89 with SC-$CO_2$ had the strongest gel structure.

An important aspect of the oscillatory results is that pH adjustment accompanied with SC-$CO_2$ by SCFX process significantly altered the gel properties of WPC. One explanation is that when the net charge on the proteins was manipulated, there were significant changes in protein-protein, and protein-solvent interactions

governed by shifting the balance of attractive and repulsive forces. This shift consequently affected the rate of aggregation, resulting in spatial arrangement of protein molecules and different gel structures depending on the extent of net charges (Clark, 1998; Tang, McCarthy, & Munro, 1995). When the SC-$CO_2$ was incorporated into the WP matrices, the porous structure of WP extrudates could be created by SC-$CO_2$ expansion at the die exit as the pressure quenched to atmospheric level. It is possible that this could cause the rapid and extensive hydration of tWPC powders even at ambient temperature. This is as would be expected if electrostatic forces are important since repulsion between like charges should lead to a more expanded and easily hydrated structure leading to stronger network as seen in some tWPC samples. Moreover, Zhong and Jin (2008) reported the improvement in gelling properties of WP upon heating after the WPC solution (10%, w/v) was treated with SC-$CO_2$ since the dissolved SC-$CO_2$ could alternate the secondary structure (α-helix, β-sheet, and random coil) of proteins (Liu, Hsieh, & Liu, 2004; Striolo, Favaro, Elvassore, Bertucco, & Di Noto, 2003). However, further studies on molecular and chemical changes of WPs induced by the SCFX process are needed to correlate with the rheological properties of tWPC products. From these findings, reactive SCFX process has been successfully integrated for texturization of WPC which exhibits cold-gelling behavior at ambient temperature.

### 2.4.6. Temperature stability of selected tWPC

Based on the viscosity and viscoelastic behavior results, tWPC samples produced under extremely acidic (pH 2.89) and alkaline (pH 8.16) conditions with SC-$CO_2$ injection were selected as the best representatives for the thermal stability and further studies. Figure 2.7 illustrates the variation of viscoelastic moduli ($G'$ and $G''$) of 20% (w/w) tWPC dispersions with temperature ramped from 25 to 85ºC.

Table 2.3. Power law parameters[a] describing the frequency dependence of moduli ($G'$ and $G''$) of dispersions containing 30% (w/w) tWPC powders.

| Extrusion pH | *p* | *q* |
|---|---|---|
| Unprocessed WPC blend (control) | -[b] | - |
| Without SC-$CO_2$ | | |
| 2.89 | 0.138 | 0.109 |
| 3.53 | 0.208 | 0.380 |
| 4.44 | 0.252 | 0.445 |
| 6.10[c] | 0.218 | 0.192 |
| 7.01 | 0.091 | 0.087 |
| 8.16 | 0.089 | 0.062 |
| With 1% (db) SC-$CO_2$ | | |
| 2.89 | 0.129 | 0.085 |
| 3.53 | 0.221 | 0.271 |
| 4.44 | 0.226 | 0.445 |
| 6.10[c] | 0.279 | 0.324 |
| 7.01 | 0.092 | 0.059 |
| 8.16 | 0.069 | 0.052 |

[a]Mean of triplicates; *p*—power law parameter relating $G'$ and $\omega$ ($G' \; a \; \omega^p$); *q*—power law parameter relating $G''$ and $\omega$ ($G'' \; a \; \omega^q$).

[b]Sample did not show the viscoelastic behavior.

[c]tWPC powder extruded with water only (non-pH-adjusted).

The same heat treatment was done to 20% (w/w) unprocessed WPC dispersion. The hard and solid gel formation was found in the unprocessed WPC after the temperature was raised beyond the gelation temperature of native WPs to 85ºC. On the other hand, both tWPC samples notably displayed little variation in $G'$ and $G''$ when the temperature was ramped from 25ºC to 85ºC. The extreme acid-treated sample showed a gradual increase in $G'$ and $G''$ as temperature was increased from 25 to 85ºC. This finding is in contrast to the results reported by Resch, Daubert, and Foegeding (2004) who found a decrease in viscosity of freeze-dried (12.2%, w/w) and spray-dried (10.2%, w/w) derivatized WPC dispersions as temperature was increased from 10 to 90ºC. These derivatized WPs have been also reported to perform a cold-setting gel at ambient temperature.

In the case of extreme alkali-treated sample, $G'$ and $G''$ appeared unchanged as the temperature was elevated from 25 to 65ºC, and slightly increased as temperature was raised to 85ºC. A slight variation in G´ and G″ of both tWPC samples after the temperature ramping could be attributed to the additional unfolding and denaturation of native WPs remaining in tWPC powders. A noticeable difference between these two samples corresponding to the temperature ramp may be explained by the difference in the magnitude of WP aggregation in these samples. Our electrophoresis SDS-PAGE results (data not shown) showed the higher extent of large WP aggregates in alkali-treated sample. This could affect the amount of native WPs remaining in tWPC powders, resulting in slight difference in their temperature correspond. As expected, SCFX process rendered WPC into an ingredient having a stable cold-set gelling behavior over a wide range of temperature.

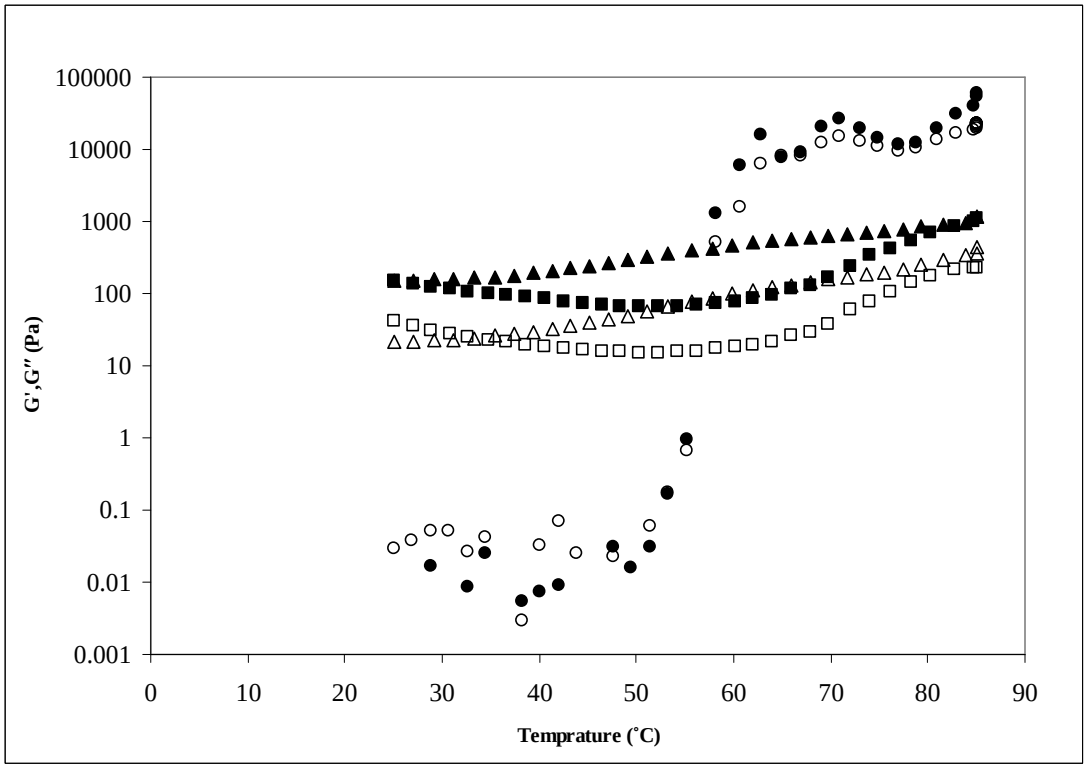


Figure 2.7. Temperature sweep test of selected WP dispersions (20%, w/w). *G′* and *G″* (▲,Δ) for dispersion containing tWPC powder extruded at pH 2.89 with 1% (db) SC-$CO_2$ injection; *G′* and *G″* (■,□) for dispersion containing tWPC powder extruded at pH 8.16 with 1% (db) SC-$CO_2$ injection; *G′* and *G″* (●,○) for WP dispersion containing unprocessed WPC blend powder. Data are averages for 3 tests. The maximum error in *G′* and *G″* data was 5%.

### 2.4.7. Water holding capacity of selected tWPC powders

It was observed that alkali-treated tWPC extrudates had turbid, opaque, and slightly dark color appearance indicating large aggregates scattering light. It was reported that this normally occurred when WPs are denatured in the alkali condition (Onwulata et al., 2006). On the other hand, the acid-treated extrudates were slightly yellowish and transparent appearance. The similar observations in WPI extruded under acidic and alkaline conditions were also reported by Onwulata et al. (2006). They indicated the differences in structure of WPI aggregates produced under different pH conditions. The pH adjustment and ionic strength could affect the rates of unfolding, degree of denaturation, and aggregate size of WPs, resulting in different rheological characteristics and the quantification of water holding in final products (Alting, 2003). It is interesting to note that injecting SC-$CO_2$ into pH-adjusted WPC yielded a lighter product color, implying the porous structure through SC-$CO_2$ expansion in WP extrudates.

tWPC samples produced under extremely acidic (pH 2.89), alkaline (pH 8.16) conditions with and without SC-$CO_2$ injection compared with the non-pH adjusted sample (pH 6.10) were selected to study their water holding capacity (WHC). The comparison of WHC values among tWPC powders is demonstrated in Figure 2.8. Without SC-$CO_2$ addition, the extreme acid-treated tWPC powder had highest WHC. This may be explained by the maximum solvent-protein interaction at extremely acidic condition (pH 2.89) due to unfolding of polypeptide chains which allowed exposure of more reactive amino acid side chains and thus, favoring water binding. At pH around 6.10 evident in non-pH adjusted tWPC, probably less protein-water interaction occurred because of the neutralized charges on amino acid side chains. Under alkaline condition, the insolubity of WPs increased due to the larger proportion of proteins being polymerized to higher molecular weight molecules and large aggregates,

resulting in turbid and particulate type of gel (Onwulata et al., 2006). According to Bowland and Foegeding (1995), Elofsson et al. (1997), and Hudson et al., (2000), particulate gels are opaque and synerese which generally have lower water holding capacity due to the large inter-particle pores. On the other hand, WPs produced under acidic conditions (pH < 3.5) were reported to form the translucent or fine- stranded type of gel with higher water holding capacity (Hudson et al., 2000; Ikeda & Morris, 2002). In addition, an increase in solubility and lower extent of aggregates of WPC produced under acidic condition was demonstrated by our protein solubility and SDS-PAGE results (data not shown). This could be a reason of lower water absorption in alkali-treated tWPC powder compared to the acid-treated tWPC powder.

Addition of 1% (db) SC-$CO_2$ significantly enhanced the WHC of tWPC powders, except for the non-pH-adjusted sample. Figure 2.8 clearly shows that the WHC of tWPC powders increased by 3 and 1.9 times for acid-treated and alkali-treated samples, respectively, compared to non pH-adjusted tWPC without SC-$CO_2$ addition. These results indicated the formation of porous structure through SC-$CO_2$ expansion in WP extrudates leading to the higher WHC and viscosity in tWPC samples as previously described.

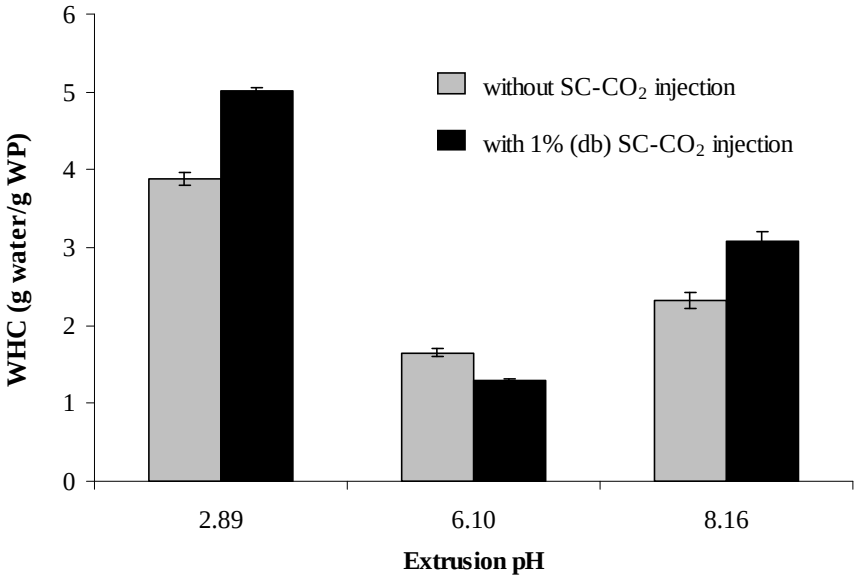


Figure 2.8. Water holding capacity (WHC) of selected tWPC powders at 25ºC. Error bars represent standard deviation from the mean of 3 trials.

## 2.5. Conclusions

The tWPC products demonstrated instant dispersibility and the ability to form a cold-setting gel without additional heat input. The 20 % (w/w) tWPC dispersions exhibited shear thinning behavior, indicating a typical characteristic of thickening agents used in food systems. Injection of 1% (db) SC-$CO_2$ to pH-adjusted protein polymer melts significantly enhanced the viscosity and other viscoselatic properties of most tWPC samples. Incorporation of SC-$CO_2$ to the extreme acid-treated tWPC showed the best rheological characteristics by contributing approximately 258 and 275,000 times higher apparent viscosity and elastic modulus than the unprocessed WPC. tWPC samples produced under extremely acidic (pH 2.89) and alkaline (pH 8.16) conditions with SC-$CO_2$ exhibited high stability of rheological properties over a wide temperature range (25 to 85 ºC). Addition of SC-CO2 also increased the water holding capacity of pH-treated tWPC samples. The results confirm our hypothesis that reactive extrusion of WPs in highly alkaline or acidic environment combined with controlled shear and heat in the presence of mineral salts ($CaCl_2$ and NaCl) and SC-$CO_2$ favorably generated new WP ingredients with unique gelling and functional properties which may open up a new avenue for utilization of WP as a thickening or gelling agent in food formulations. Further studies are underway to understand the mechanisms of molecular and chemical changes of WPs induced by the SCFX process which could be correlated with the enhanced rheological properties of tWPC products.

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# CHAPTER THREE

# PHYSICAL AND CHEMICAL CHANGES IN WHEY PROTEIN CONCENTRATE TEXTURIZED BY REACTIVE SUPERCRITICAL FLUID EXTRUSION*

## 3.1. Abstract

The mechanisms of interactions in whey protein concentrate (WPC) texturized by reactive supercritical fluid extrusion and pH modifications were evaluated in terms of protein solubility in different extraction buffers, electrophoresis, free sulfhydryl (SH) groups, and apparent viscosity. The soluble protein content and free SH groups of the texturized WPC (tWPC) produced at pH 2.89 decreased by ~20% and 16% relative to the unextruded control. It was completely soluble in the presence of urea and SDS, indicating the importance of non-covalent interactions in maintaining the structure of this product. Its dispersion (20% w/w) yielded a creamy texture with a particle size in the micron-range (mean diameter ~5 μm) and contributed ~258 times higher viscosity compared to the unextruded control. The tWPC produced at pH 8.16 was soluble only in the presence of a reducing agent. It yielded a grainy texture with a high proportion of large particles due to an extensive aggregation via intermolecular disulfide formations.

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### 3.2. Introduction

The extrusion process has been utilized to alter whey protein (WP) conformational structures and functionalities for providing protein enriched food products with a wide variety of textures (Martinez-Serna & Villota, 1992; Tunick & Onwulata, 2006). In general, proteins are susceptible to both conformational changes and chemical reactions during extrusion (Wasserman et al., 1992). The combination of shear, temperature, and pressure during extrusion processing creates opportunities for protein molecular transformations (Ledward & Tester, 1994; Yuryev et al., 1990). The increase in pressure and temperature as a result of both transfer of heat from the heated barrel and the conversion of mechanical energy into heat energy accompanied with the shearing and mixing of the extruder screw causes protein denaturation, which exposes the reactive free sulfhydryl (SH) groups, non-polar amino acids, and peptides that are normally concealed in the native proteins (Kim & Maga, 1987; Wasserman et al., 1992).

Ledward and Mitchell (1988) proposed that during the extrusion process proteins possibly (1) form randomly aggregated or oriented spherical molecules, or (2) aggregate as strands, either randomly or oriented in the direction of flow. However, the effects of extrusion on the molecular changes of WPs are still difficult to isolate because high protein concentrations are exposed to several processes simultaneously. Only few investigators have addressed the mechanisms of WPs reactivity during extrusion, especially at high levels of protein concentrations in the limited water content (Martinez-Serna & Villota, 1992). Studies on texturization of soy, wheat, and whey proteins have attributed to a combination of fragmentation and aggregation, non-covalent associations, and covalent cross-linking (Akdogan, 1999; Areas, 1992; Burgess & Stanley, 1976; Rebello & Schaich, 1999). Early studies on extrusion of soy proteins claimed that new peptide bonds were responsible for the extrudate structure

and disulfide (S-S) bonds had less significant impact on it (Burgess & Stanley, 1976). However, Areas (1992), Hager (1984), Ledward and Tester (1994), and Yuryev et al. (1990) concluded that S-S bonds, non-specific hydrophobic and electrostatic interactions are all responsible for protein texturization by extrusion.

The effects of pH modifications during extrusion process on WP reactivity and functionality of final products have been reported (Amaya-Llano et al., 2007; Dahl & Villota, 1991; Martinez-Serna & Villota, 1992; Onwulata et al., 2006; Law & Leaver, 2000; Monahan et al., 1995; Walsh & Carpenter, 2001). Their studies showed that conformations and protein-protein interactions of WPs were strongly affected by pH. In general, the alkaline treatments decreased protein solubility due to extensive S-S bond formations. The resulting products had a fibrous structure and could be used as meat alternatives. Acidic treatments, on the other hand, showed higher protein solubility and produced non-oriented fiber arrangement of extruded proteins (Dahl & Villota, 1991; Onwulata et al., 2006). Queguiner et al. (1992) also reported that the microparticulated whey protein isolate (WPI) could be obtained by extrusion at acidic pH (pH 3.9). The resulting product displayed a semi-solid, smooth texture and contained a high proportion of small particles (mean diameter ~11.5 μm).

Our current research involves the texturization of whey protein concentrate (WPC) using reactive supercritical fluid extrusion process (SCFX). Adjustment of pH through acid or alkali addition during reactive SCFX was shown to greatly influence the rheological properties of commercial WPC-80 (Manoi & Rizvi, 2008). The resulting texturized WPC (tWPC) produced under highly acidic (pH 2.89) or alkaline (pH 8.16) condition exhibited a dramatic increase in viscosity and water holding capacity (Manoi & Rizvi, 2008). Undoubtedly, within the extruder, the physical and chemical changes are expected to dramatically alter the degree of reactivity and conformations of WPs with subsequent effects on the functional properties of the final

tWPC products. Thus, the main focus of this study was to investigate the mechanisms of interactions of WPs during reactive SCFX in highly acidic (pH 2.89) and alkaline (8.16) conditions and to elucidate their influences on the selected physicochemical properties of the final tWPC products. The contribution of covalent (disulfide bonds) and non-covalent (hydrophobic and hydrogen bonds) interactions in tWPC samples were evaluated and compared with the extruded (non-pH-adjusted) and unextruded controls on the basis of protein solubility in different extraction buffers, SDS-PAGE, and sulfhydryl (SH) group content. The protein aggregate size and apparent viscosity of tWPC and the control powders were also determined.

## 3.3. Materials and methods

### *3.3.1. Materials and feed formulation*

Commercial WPC-80 was obtained from Leprino Foods Company (Lemoore West, CA, USA). Sodium hydroxide (NaOH) and hydrochloric (HCl) solutions were used as the pH-adjusting agents. All chemicals used were analytical grade (Sigma Chemical Co., St. Louis, MO, USA). A feed formulation consisted of (by weight) 94% pre-hydrated (10% dry basis) WPC-80, 6% pre-gelatinized corn starch (Cerestar USA, Inc., Hammond, IN, USA), 0.6% (WPC-starch basis) $CaCl_2$, and 0.6% (WPC-starch basis) NaCl. In this process, the pre-gelatinized corn starch was used as a binder to hold protein matrices because of their ability to form hydrogen bonds in the extruded product (Amaya-Llano et al., 2007). Earlier studies indicated that adding a minimal content of pre-gelatinized corn starch in the WPC blend would facilitate the manufacturing of tWPC extrudates (Manoi & Rizvi, 2008). Results showed that starch-protein interactions did not significantly influence the rheological properties of final tWPC products since starch acted as an inactive filler in the tWPC formation (Manoi & Rizvi, 2008).

*3.3.2. Production of tWPC by reactive SCFX process*

Extrusion-texturization of WPC-based formulation was performed on a pilot-scale Wenger TX-52 Magnum (Wenger Manufacturing, Sabetha, KS, USA) co-rotating twin-screw extruder with a length to diameter ratio (L/D) of 28.5. Extrusion conditions were: screw speed, 180 rpm; temperature profile in the barrel towards the die plate, 25º, 90º, 90º, 90º, 90ºC; die diameter (two circular inserts), 1.2 mm; dry feed rate, 35 kg/h. The average specific mechanical energy (SME) input for the process was 57 Wh/kg. The HCl (15% v/v) or NaOH (1.67% w/v) solution was injected to the extruder at the mixing zone to create pH of 2.89 or 8.16 and water stream was used as a control (extruded control). Extrusion was conducted at 60% (dry feed basis) moisture content. A pilot scale supercritical fluid system was used for injecting SC-$CO_2$ into the pH-treated protein polymer melts at L/D of 24 through four injection valves located around the extruder barrel. The die pressure was maintained higher than pressure inside the barrel for continuous SC-$CO_2$ flow into the protein polymer melt at the desired rate (1% dry feed basis) and pressure (10-15 MPa). Product temperatures were measured by a thermocouple at the end of the extruder. The tWPC extrudates were collected and dried at room temperature overnight or until their moisture contents were between 5 to 7%. Dried tWPC extrudates were ground using a mill machine (Thomas-Wiley Mill model ED-5, Arthur H. Thomas Co., PA, USA) to reduce the particle size to less than or equal to 125 μm. All samples were then stored at room temperature in air tight containers until analyzed.

*3.3.3. Protein solubility*

Solubility of unextruded control, extruded control, and tWPC powders was determined according to Shimada and Cheftel (1989) with some modifications. The powders were extracted with three different types of buffers: (a) standard buffer

containing 0.086 M Tris, 0.09 M glycine, and 4 mM ethylenediaminetetraacetic acid disodium salt ($Na_2EDTA$), pH 8.0, (b) standard buffer containing 8 M urea and 0.5% (17.3 mM) sodium dodecyl sulfate (SDS), pH 8.0, and (c) standard buffer containing 8 M urea, 0.5% SDS, and 10 mM dithiothreitol (DTT), pH 8.0. The protein solutions were adjusted to 0.1% w/v (0.05 g of protein/50 ml of buffer), mixed at room temperature for 1 h, and then centrifuged at 19,000 g for 15 min at 20ºC. The soluble protein content of the supernatant was determined using the bicinchoninic acid (BCA) protein assay kit from Pierce (Rockford, IL, USA). Protein solubility expressed as percentage of total protein was evaluated and the mean of three replicates was reported.

*3.3.4. Polyacrylamide gel electrophoresis*

Protein samples were analyzed for hydrolysis by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with a NuPAGE® Electrophoresis System (Invitrogen, California, USA). All procedures were followed according to the NuPAGE® Novex Bis-Tris Gel Instruction Card (IM-8042). The samples were dissolved in the buffer solution composed of deionized water, 20 mM Tris, 5mM EDTA, and 2.5% SDS. The soluble protein contained in a supernatant was used for the SDS-PAGE analysis. Samples (10 µg proteins on each lane) were run under non-reducing and reducing conditions using a 12% NuPAGE® Novex Bis-Tris separating gel and 4% stacking gel with 1 mm thickness and 10 well format (Invitrogen, California, USA). Under the reducing conditions, dithiothreitol (DTT) was added to protein solutions as a reducing agent and then the samples were boiled at 70 ºC for 10 min before loading to the gel. The NuPAGE® MOPs SDS running buffer (20X) containing 50 mM MOPS, 50 mM Tris base, 0.1% SDS and 1mM EDTA (pH 7.7) was used. The gels were run for 35 min at a constant voltage of 200 V and an initial current of 125 mA per gel. The gels were stained with a Coomassie Blue solution

(Invitrogen, California, USA). Following staining, the gels were destained with multiple changes of 40% methanol, 10% glacial acetic acid solution. The wide range marker12 from Invitogen was used as the markers; 200 kDa Myosin, 116.3 kDa beta-galactosidase, 97.4 kDa Phosphorylase B, 66.3 kDa BSA, 55.4 kDa Glutamic dehydrogenase, 36.5 kDa Lactate dehydrogenase, 31.0 kDa Carbonic anhydrase , 21.5 kDa Trypsin inhibitor, 14.4 kDa Lysozyme, 6.0 kDa Aprotinin, 3.5 kDa Insulin B chain, and 2.5 kDa Insulain A chain.

*3.3.5. Free sulfhydryl (-SH) group*

The free SH group content of protein solutions was determined with use of Ellman's reagent, DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid) according to Sava et al. (2005) and Shimada and Cheftel (1989) with some modifications. Protein solutions were diluted (each at 0.1% w/v protein concentration) with the solubilized buffer (0.086 M Tris, 0.09 M glycine, 4 mM $Na_2EDTA$, 0.5% SDS, 8 M urea, pH 8.0). These samples were mixed at room temperature for 1 h, and then centrifuged at 19,000 g for 15 min at 20 ºC. 0.03 ml of DTNB solution (40 mg of DTNB/10 ml of standard buffer) was added to a 3-ml aliquot of the protein supernatant. The absorbance at 412 nm was measured against a reagent blank at 15 min on a Spectronic 1200 spectrophotometer (Bausch and Lomb, Rochester, NY, USA) at 20 ºC. A molar extinction coefficient of $1.36 \times 10^4$ $M^{-1}$ $cm^{-1}$ was used to calculate the amount of free SH groups, expressed in µmoles per gram of protein. The mean of three replicates was reported.

*3.3.6. Particle size distribution*

The effective diameters of protein particles in suspension (0.1%, w/w) were determined using 90 Plus Particle Size Analyzer (Brookhaven Instruments

Corporation, Holtsville, NY, USA), which was calibrated beforehand using a standard solution with particles of known uniform size. The instrument was equipped with MAS OPTION particle sizing software. Measurements were done in triplicate per protein solution preparation.

*3.3.7. Apparent viscosity*

The protein powders were reconstituted at 10-20% (w/w) concentration in deionized water and gently stirred for 2 h, and then stored overnight at 4 ºC prior to testing. The parallel plate geometry with 50 mm plate diameter was utilized for steady shear rate ramp test. The sample was loaded into the rheometer (ARES strain-controlled rheometer, TA Instruments, New Castle, DE, USA) equipped with a Peltier temperature controlling system, and then the top plate was slowly lowered until the final sample thickness of 1 mm was achieved. A thin layer of mineral oil was applied to the exposed sample edges to prevent moisture loss. All experiments were conducted at 25 ºC. Shear rate was ramped from 1 to 100 $s^{-1}$. The apparent viscosities were recorded by TA Orchestrator software. All determinations were done in triplicates.

*3.3.8. Confocal laser scanning microscopy (CLSM)*

The selected protein samples were stained with Fast Green FCF (0.001%, w/w in deionized water). The stained sample was placed on a glass slide and covered with a cover slide. The CLSM was performed on a Leica TCS-SP2 Confocal Laser Scanning head mounted on a Leica DMRE-7 (SDK) upright microscope (Leica Microsystems Inc., Bannockburn, IL, USA) equipped with a 20x HC PL APO/ 0.70NA oil immersion objective lens. 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 green emission range was 500-580 nm and the red emission range was 650-730 nm.

## 3.4. Results and discussion

### *3.4.1. Protein solubility*

Solubility is an important property due to its significant influence on functional properties of proteins such as emulsifying, foaming, whipping, and gelling properties (Nakai & Chan, 1985). A decrease in protein solubility affects the protein functionality (Pelegrine & Gasparetto, 2005). The solubility depends on whether the proteins are in their native or denatured state but denaturation alone is not enough to cause measurable loss of solubility, as the protein must also aggregate (Anandharamakrishnan et al., 2008). Solubility of proteins relates to covalent (disulfide linkage) and surface hydrophobic (protein-protein) and hydrophilic (protein-solvent) interactions (Liu & Hsieh, 2007; Pelegrine & Gasparetto, 2005).

The solubility in different extraction buffers of tWPC samples produced at pH 2.89 and 8.16 compared with that of unextruded and extruded (non-pH-adjusted) controls is presented in Figure 3.1. The unextruded control exhibited the highest protein solubility (~90%) in standard buffer because it was mainly composed of proteins in the native state.

However, it was observed that the solubility of WPs generally decreased after extrusion. Results shown in Figure 3.1 indicate that the standard buffer extracted the least amount of proteins in tWPC samples as well as the extruded control. The soluble protein content in the tWPC produced at pH 2.89, the extruded control, and the tWPC produced at pH 8.16 decreased by approximately 23%, 45%, and 78%, respectively, relative to the unextruded control (Figure 3.1). Li and Lee (1996) mentioned that the aggregation of proteins during extrusion processing is responsible for a decrease in their solubility. The aggregation of proteins is heavily influenced by pH as it affects the net charge on the protein molecules, and thus the electrostatic repulsive forces

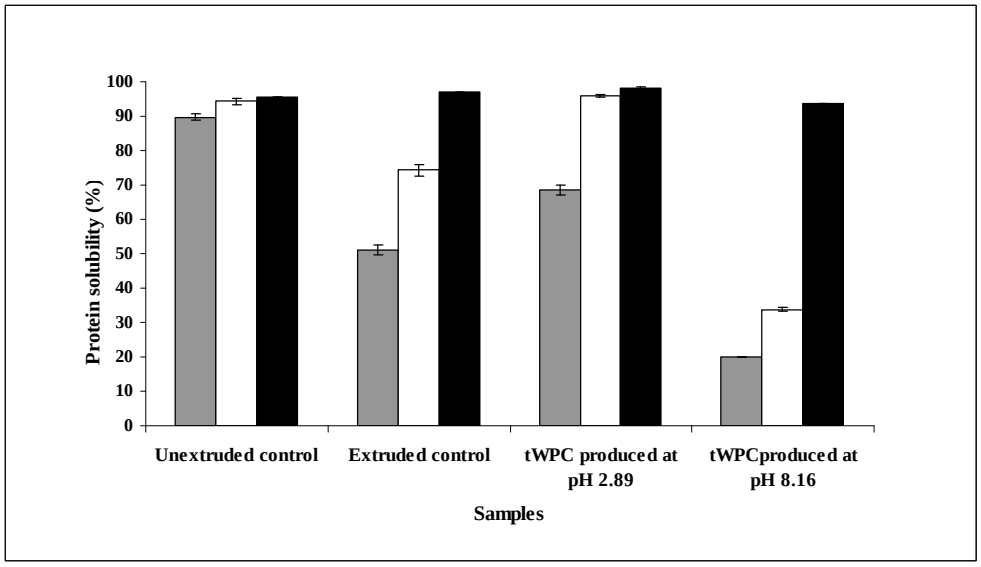



Figure 3.1. Protein solubility (expressed as percentage of total protein) of unextruded control, extruded control, and tWPC samples produced at pH 2.89 and pH 8.16. Extraction solutions: (▩) standard buffer (0.086 M Tris, 0.09 M glycine, and 4 mM ethylenediaminetetraacetic acid disodium salt ($Na_2EDTA$), pH 8.0), (□) standard buffer (pH 8.0) containing 8 M urea and 0.5% SDS, and (■)standard buffer (pH 8.0) containing 8 M urea, 0.5% SDS, and 10 mM dithiothreitol (DTT). (Mean ± SD, n = 3).

between molecules (Anandharamakrishnan et al., 2008). The result clearly shows that the tWPC produced at pH 2.89 exhibited a much higher solubility (~70%) in standard buffer as compared to a pronounced minimum solubility (~20%) of tWPC produced at pH 8.16. Onwulata et al. (2006) demonstrated similar results for WPI extruded under acidic (pH 1.4-2.5) and alkaline (pH 11.5-13.2) conditions. The authors explained that the denaturation of WPs was more extensive under alkaline conditions which subsequently resulted in a decrease of protein solubility. In addition, deWit and Klarenbeek (1984) and Monahan et al. (1995) stated that generally the β-lactoglobulin (β-Lg), the major protein in whey, is more rigid and more heat stable at acid (pH ≤ 3.0) than at neutral or alkaline pH. Diluted solutions of WPs and especially of β-Lg were reported to be less heat-aggregates and higher soluble at acidic pHs (2.0-3.5) than at higher pH (4.5-6.5) (Harwalkar, 1980a; Modler & Emmons, 1977; Shimada & Cheftel, 1989).

The solubility of protein samples in standard buffer containing urea and SDS is also presented in Figure 3.1. Urea is an agent known to disrupt non-covalent interactions, such as hydrogen bonds and hydrophobic interactions, while SDS is expected to associate with hydrophobic amino acids in the protein and thus interfere with hydrophobic protein-protein interactions (Otte et al., 2000; Shimada & Cheftel, 1989). Results shown in Figure 3.1 clearly indicated that approximately 96% of total proteins in tWPC produced at pH 2.89 became soluble in the presence of urea and SDS. It is likely that mainly non-covalent interactions (hydrophobic interactions and hydrogen bonds) are responsible for the maintenance of the structures of this tWPC. On the basis of the above results, the change in WP solubility during reactive SCFX under the acidic condition of pH 2.89 possibly involves the two-step processes of: (1) unfolding and exposing hydrophobic and reactive sites of molecules and (2) aggregating and increasing the molecular weight, which resulted in the loss of protein

solubility. Queguiner et al. (1992) produced microparticulated WPs by extruding WPI powder in the pH range of 3.5-3.9 at 77% moisture content and temperature range of 90-100 °C. Their results showed that the resulting microparticulated WP product displayed high nitrogen solubility (43-47%) and it was completely soluble in the buffer containing 1% SDS. The authors concluded that under the acidic condition the hydrophobic and hydrogen bonds mainly involved in the structural formation of microparticulated WP. Our results revealed that non-covalent bonds were also presented in the unprocessed WPC. This would be expected since the commercial WPC product had undergone heat treatment during the spray drying process which could induce partial denaturation and insolubility of proteins (Anandharamakrishnan et al., 2008). The increase in protein solubility in standard buffer containing urea and SDS was also observed for extruded control (~74%) and tWPC produced at pH 8.16 (~33%). This observation indicates that hydrophobic interactions were partially involved in maintaining the structure of these samples, but to a lesser extent than in the tWPC produced at pH 2.89.

The differences between the solubility of proteins in standard buffer containing urea and SDS with and without a reducing agent (DTT) are also presented in Figure 3.1. DTT is a strong reducing agent and generally can cleave S-S bonds. Thus, the comparison of the protein solubility in both extraction buffers provides information about protein cross-linking by intermolecular S-S formations. With the presence of DTT in the extraction buffer, the content of soluble proteins in all samples was very close to the total protein in the initial protein solution and a near equal amount of extractable proteins in all protein samples was observed (Figure 3.1). Our results showed that the intermolecular S-S bonds were the most abundant in tWPC produced at pH 8.16 indicated by a drastic increase in the solubility of proteins from ~33% in the buffer without DTT to ~93% in the buffer with DTT. This indicated that WPs

extensively aggregated through intermolecular S-S interactions during reactive SCFX under the alkaline condition and consequently resulted in a decrease in solubility. Similar observations have been reported for WPI-corn starch extrudates (Martinez-Serna & Villota, 1992). The authors stated that through increasing the negative charge of proteins by increasing the pH, S-S bonds were more involved in the extrudates. Furthermore, Hoffmann and van Mil (1999) reported that in the pH range of 6.4-8.0, β-Lg aggregates were formed mainly by intermolecular S-S bonds.

Studies on molecular mechanisms of protein interactions during extrusion processing of various types of proteins including wheat flour, soy, and whey have been well discussed (Fisher, 2004; Hager, 1984; Li & Lee, 1996; Liu & Hsieh, 2007; Martinez-Serna & Villota, 1992; Prudêncio-Ferreira & Arêas; 1993). The authors proposed that both the S-S bond formation and the hydrophobic interaction were responsible for the aggregation of proteins. Our present results revealed that the mechanisms of protein-protein interactions during reactive SCFX were highly dependent on the pH conditions of the system. The major forces responsible for the change in solubility and conformational structure of WPs during reactive SCFX appear to be non-covalent bonds (hydrophobic interactions, hydrogen bonds) and covalent bonds (S-S bonds). The formation of intermolecular S-S bonds was more extensive in the tWPC produced under the alkaline condition at pH 8.16, while the hydrophobic interactions were less important. On the other hand, the tWPC produced under the acidic condition at pH 2.89 primarily consisted of hydrophobic and hydrogen bonds, and the S-S bond was partially responsible for the protein structures. The proposed mechanism of protein-protein interactions under acidic and alkaline conditions during reactive extrusion is demonstrated in Figure 3.2.

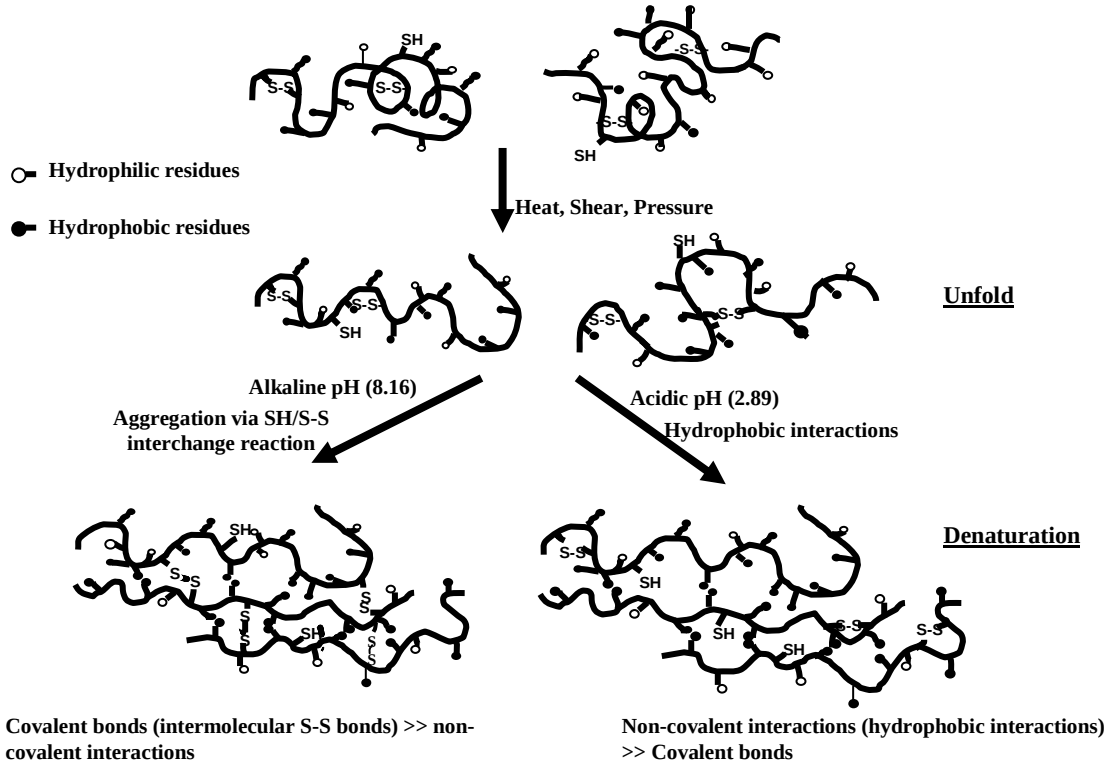



Figure 3.2. Protein-protein interactions during reactive extrusion under acidic and alkaline conditions.

*3.4.2. SDS-PAGE*

SDS-PAGE of soluble protein fractions in the unextruded control, extruded control, and tWPC samples was studied under reducing and non-reducing conditions to estimate the molecular weight distribution and S-S bond cross-linking of proteins. An equal amount of the supernatant was loaded on each well of the gels to compare the changes in the relative molecular weight distribution of each sample. Under the non-reducing condition, there was no pronounced difference in SDS-PAGE patterns of the unextruded control (lane 2) and the tWPC produced at pH 2.89 (lane 4) (Figure 3.3). This indicated that a large proportion of protein monomers in the soluble fraction of tWPC produced at pH 2.89 (lane 4) was recovered by SDS. It confirmed the solubility results that the non-covalent interactions dominated the structural formation of tWPC produced at pH 2.89. A similar observation was reported in acidified WPC gels produced at 75ºC in the pH range of 2.0-4.5 (Boye et al., 1995). The authors showed that the electrophoretic patterns of the acid WPC gels were similar to those of native WPC and the BSA, β-Lg, and α-La bands could still be detected. Mills and Creamer (1975) also reported a drastic change in the surface hydrophobicity of β-Lg under acidic conditions compared to alkaline conditions. They described that the changes of proteins at acidic pH were attributed to noncovalent monomer-dimer transitions rather than to substantial changes in protein secondary structure. Our preliminary studies on the differential scanning calorimetry (DSC) of the tWPC at pH 2.89 showed no endothermic reaction (data not shown), indicating it was a product of an irreversible denaturation. Harwalkar (1980a,1980b) explained that thermal denaturation of β-Lg at pH 2.5 did not change the molecular size of proteins but altered their molecular structure as determined by the specific optical rotation $[\alpha]_{589}^{25}$, electrophoretic analysis and measurements of sedimentation velocity. In addition, the soluble fraction from the sample was similar to native β-Lg obtained by the above

methods. On the other hand, the non-reduced SDS-PAGE of the extruded control (lane 3, Figure 3.3) revealed a pronounced decrease in the intensity of the monomeric BSA, β-Lg, and α-La bands. It suggests the aggregation between BSA, α-La and β-Lg through covalent bonds during reactive extrusion of WPs at neutral pH. The non-reduced SDS-PAGE of the tWPC produced at pH 8.16 (lane 5, Figure 3.3) displayed a diminished band for the high molecular weight protein aggregates that were not solubilized by SDS. These very large molecular weight aggregates were unable to penetrate the pores of the separating gel, indicating the polymerization of proteins through covalent S-S bonds during reactive SCFX under the alkaline condition. This finding agrees with the work of Boye et al. (1995) who reported the complete disappearance of β-Lg and α-La bands at higher pH (6.0-10.0). Hoffmann and van Mil (1999) also observed the high molecular mass aggregates on top of the gel after β-Lg was heated at pH 6.4-8.0.

Under the reducing condition (Figure 3.4) the intensity of protein bands was noticeably recovered in all samples. Results reported here parallel the protein solubility assays in that all samples were completely solubilized with the presence of DTT, indicating the importance of intermolecular S-S bonds in stabilizing the tWPC structures. In this respect, a significant proportion of protein materials solubilized into monomers in the presence of SDS and DTT reflecting the sum of non-covalent and covalent aggregates. Comparison of SDS-PAGE under non-reducing and reducing conditions (Figure 3.3 and 3.4) clearly showed that intermolecular S-S bonds were more extensive in the tWPC produced at pH 8.16. However, no complete dissociation of the aggregates into monomers/dimers was observed in this sample. The intensity of protein monomers in this sample (lane 5, Figure 3.4) was still relatively low compared to the other protein samples. The losses of protein monomers observed in the tWPC produced at pH 8.16 may have resulted mainly from the degradation of some S-S

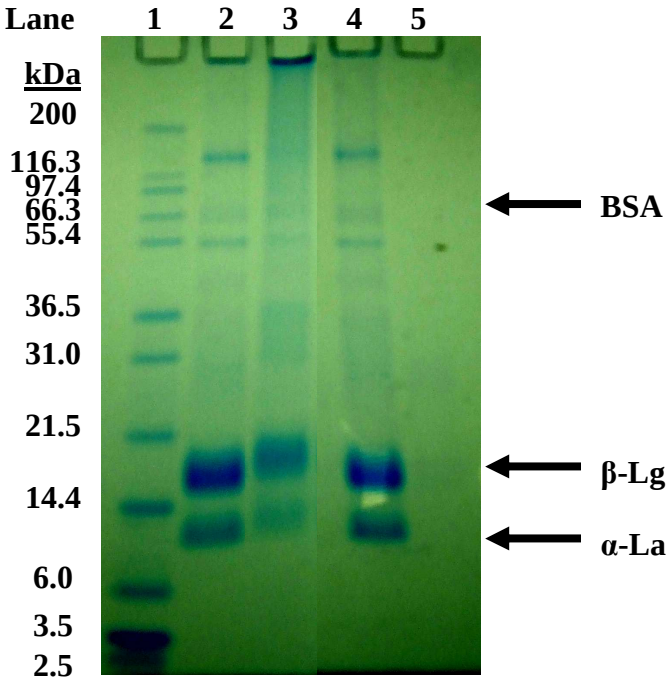



Figure 3.3. SDS-PAGE of soluble protein fractions from unextruded control (lane 2), extruded control (lane 3), and tWPC samples produced at pH 2.89 (lane 4) and pH 8.16 (lane 5) under a non-reducing condition. Lane 1 is the protein markers.

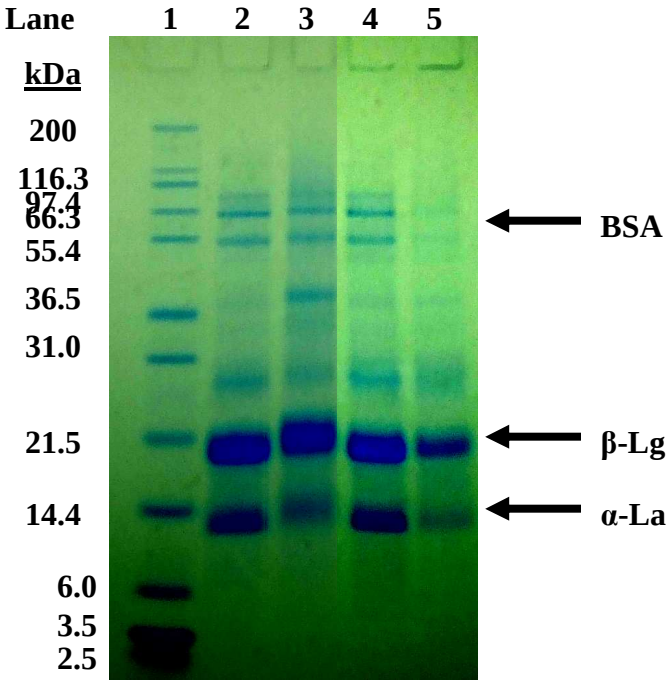



Figure 3.4. SDS-PAGE of soluble protein fractions from unextruded control (lane 2), extruded control (lane 3), and tWPC samples produced at pH 2.89 (lane 4) and pH 8.16 (lane 5) under a reducing condition. Lane 1 is the protein markers.

bonds through hydrolysis, α-elimination, and β-elimination mechanisms (Nashef et al., 1977). Destruction of these essential S-S bonds invariably led to loss of protein conformation, reactivity, and bioavailability (Florence, 1980; Robbins & Ballew, 1982). This could affect the DTT binding and SDS-PAGE patterns for this protein sample.

*3.4.3. Free sulfhydryl (SH) group*

The total free SH group contents of soluble protein fractions obtained from the unextruded control, extruded control, and tWPC samples are presented in Figure 3.5. The content of free SH groups decreased slightly from 60.75 μmol/g protein in the unprocessed WPC to 49.85 μmol/g protein in the tWPC produced at pH 2.89. Thus it is likely that during reactive SCFX under the acidic condition, the low reactivity of free SH groups in WPs diminished the likelihood of disulfide-mediated polymerization. This would agree with the protein solubility and the SDS-PAGE results showing that the non-covalent interactions dominated the structural formation of tWPC produced at pH 2.89 over S-S bonds. Queguiner et al. (1992) speculated that at acidic pHs (2.5-3.5), the low SH reactivity prevents SH/S-S interchange reactions and the formation of intermolecular S-S bonds. Similar observations were also reported in acidified gels prepared with WPI (Lupano et al., 1992; Shimada & Cheftel, 1988), WPC (Lupano et al., 1996), and soy protein isolates (SPI) (Puppo et al., 1995). Their observations indicated that the SH group in acidified WP gels remained identical with those of unheated WP, and no additional S-S bonds were formed. In addition, Monahan et al. (1995) found that under acidic conditions (pH 3.0 and 5.0) the total SH content of soluble WPI solutions or gels did not change with heating up to 90 ºC. This could be explained that by the reactivity of SH groups, which enhances both the oxidation of SH groups into S-S bonds and SH/S-S interchange reactions, decreases

significantly under acidic conditions. Free SH groups from WPs were reported to be released and to have very high reactivity under certain pH and temperature (Bazinet et al., 1997; Dunnill & Green, 1965).

In contrast, a dramatic change in free SH groups was revealed in the extruded control and the tWPC produced at pH 8.16 (Figure 3.5). The result shown in Figure 3.5 indicates the lowest free SH content (25.84 μmol/g protein) in the tWPC produced at pH 8.16. Monahan et al. (1995) reported the low SH content of WP solutions at alkaline pH (9 and 11) due to the polymerization of WPs through SH-SH oxidation and SH/S-S interchange reactions. The SH groups in the reactive $PS^-$ form can oxidize into S-S bonds or participate in SH/S-S interchange reactions (Shimada & Cheftel, 1988). Our results imply the increased propensity of WPs to undergo S-S-mediated polymerization reactions during extrusion under the alkaline pH compared to the acidic pH. It also attributes an increase in the frequency of SH/S-S interchange reactions in the unfolded proteins under the heat and alkaline treatments.

However, the decrease in SH content may also be due to degradation reactions as shown in the SDS-PAGE. It was indicated that under alkaline conditions cystine and cysteine residues may be converted into $H_2S$ and dehydroalanine (Nashef et al., 1977). Watanabe and Klostemeyer (1976) showed that the formation of degradation products from cysteine and cystine significantly contributed to the reduction of SH group content at increasing pH. The parallel changes between soluble free SH groups and the soluble protein content in the WP extrudates support our previous conclusion that S-S bonds primarily contribute to the structure of tWPC prepared at alkaline pH. The aggregations of proteins at alkaline pH resulted in an increase in their molecular weights as shown in the SDS-PAGE results and, subsequently a decrease in solubility.

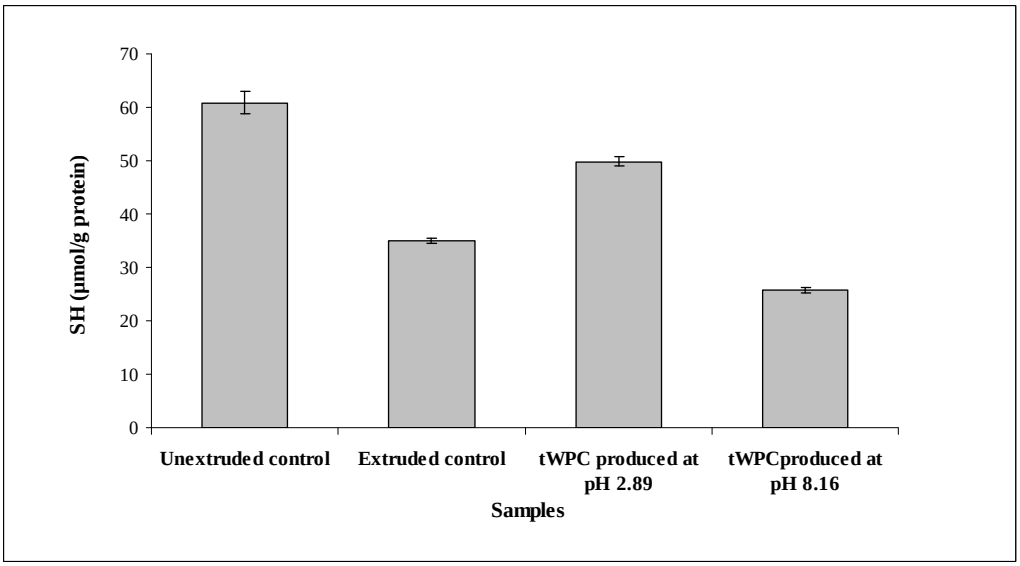



Figure 3.5. Sulfhydryl (SH) group content of the soluble protein fraction obtained from unextruded control, extruded control, and tWPC samples produced at pH 2.89 and pH 8.16. (Mean ± SD, n = 3).

*3.4.4. Apparent viscosity*

The variation of apparent viscosity (at 61.15 $s^{-1}$ shear rate) with whey protein concentrations (10 to 20% w/w) at 25 °C for unextruded control, extruded control, and tWPC dispersions is illustrated in Figure 3.6. The protein concentration did not largely influence the apparent viscosity of the unextruded control. This is because it is mainly composed of proteins in the native state (folded globular shape) which usually will not achieve a high viscosity, even at moderately high concentrations (Clark, 1998).

The apparent viscosity of the extruded control slightly increased with the increase in protein concentration. On the other hand, a drastic increase in apparent viscosity was observed in both tWPC samples. The tWPC samples exhibited the ability to impart a wide range of viscosities by varying protein concentrations. This corresponds to stronger protein-protein and hydrodynamic interactions among particles with an increase in solids in the system (Dahl & Villota, 1991).

Results shown in Figure 3.6 clearly indicated that the tWPC produced at pH 2.89 yielded the highest apparent viscosity at equal protein concentrations. At 20% (w/w) WP dispersion, it exhibited a highly viscous and creamy texture. Its microscopic structure compared with the unextruded control at 25 °C and equal protein concentration (20% w/w) was demonstrated by the confocal laser scanning microscope (CLSM) (Figure 3.7). The confocal images clearly showed that the tWPC produced at pH 2.89 formed a particulate gel structure made of protein aggregates with random sizes, while the unextruded control exhibited a homogeneous solution. The particle size distribution of tWPC produced at pH 2.89 in suspension (0.1% w/w) compared to that of unextruded control was determined and presented in Figure 3.8. The resulting particle sizes of the tWPC produced at pH 2.89 were in the range of 3.5 to 7.5 μm with the mean diameter ~ 5 μm, while the unextruded control contained the

much smaller particles with the mean diameter ~ 0.2 μm. The particle swelling caused by hydrogen bonds between amino acid groups and water, resulting in an increase in the molecular radii of protein molecules could considerably be involved in the viscosity increase in the tWPC sample.

It was interesting to note that the particulate gel structure of the tWPC produced at pH 2.89 was the inevitable outcome of the extensive acid-induced denaturation and polymerization of proteins mainly through non-covalent interactions during reactive SCFX. Rector (1992) speculated that when proteins were denatured they were usually packed closely together. This promoted protein-protein interactions, greater protein molecular entanglements, and polymerization, all of which contributed to the viscosity increases (Rattray & Jelen, 1995). This is in accordance with the studies of Otte et al. (2000). The authors reported that at low pH, the strong acidified gel could be formed when the electrostatic repulsive forces between positively charged amino acid groups are believed to be balanced by attractive hydrogen or hydrophobic bonds with the presence of optimum S-S linkages. Lieske and Konrad (1994) mentioned that hydrophobic forces were the non-covalent forces mainly responsible for the molecular arrangement of β-Lg in the microparticulated WP prepared under an acidic condition. This product is prepared by thermocoagulation process of a 40% (w/w) WPC dispersion under acid conditions (pH 3.7-4.2) with a shear rate of approximately 500,000 $min^{-1}$ in the presence of 3% lecithin (Singer et al., 1990). The resulting product had a particle size between 0.5 to 3.0 μm, behaved like a thick fluid, had a mouth feel of a creamy fat, and could be used as a protein-based fat substitute (Kulozik et al., 2001; Lieske & Konrad, 1994). In addition, Queguiner et al. (1992) reported that the acid-treated WPI (pH 3.9, 20% protein) produced by a twin-screw extruder at 90-100 ºC exhibited a firm, cohesive, spreadable consistency, and a smooth texture with small coagulated particles (mean diameter ~11.5 μm).

The tWPC produced at pH 8.16 was also observed to impart high viscosity with protein concentrations. It is likely that the large molecular weight protein aggregates in this sample were found to have increased viscosity. The larger aggregates generally possess a greater effective volume which could entrap more water than the individual small WP molecules (Firebaugh, 2004). However, the tWPC produced at pH 8.16 exhibited a poor solubility and yielded a gritty texture with clearly visible coarse particles which prevented a continuous gel formation. The size distribution results for the tWPC produced at pH 8.16 in suspension are not included due to the formation of overly large aggregates in this sample which limited the instrument capability and accuracy.

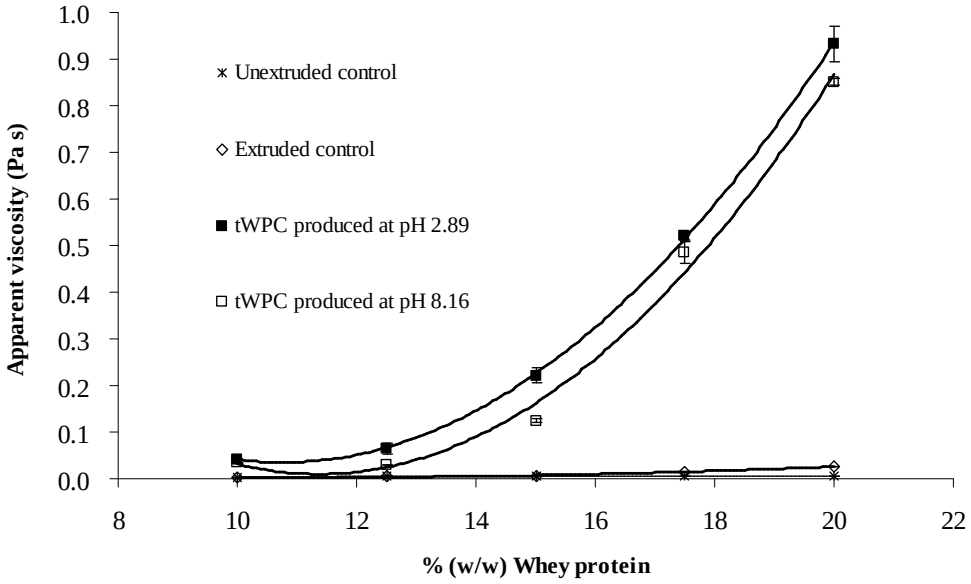
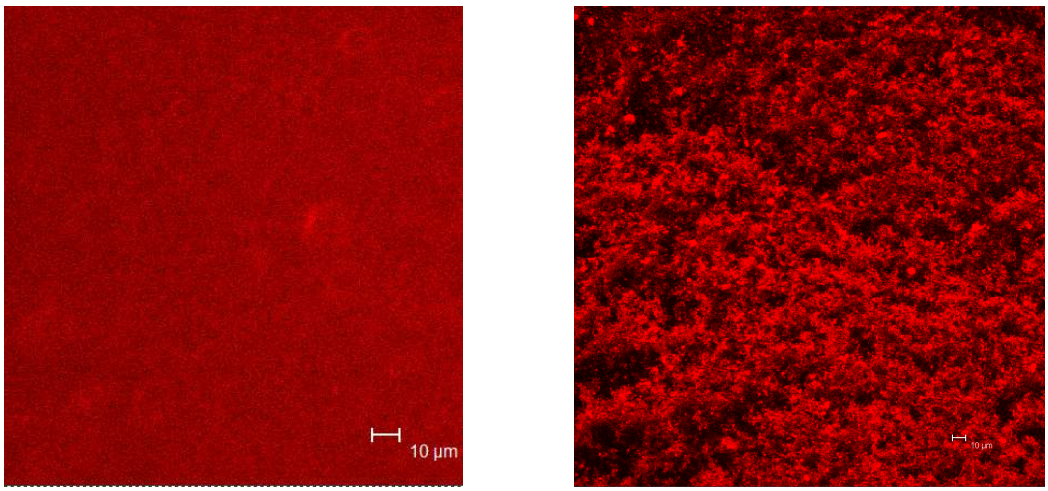



Figure 3.6. Variation of apparent viscosity (at 61.15 $s^{-1}$ shear rate) with whey protein concentration for unextruded control, extruded control, and tWPC dispersions at 25 °C. (Mean ± SD, n = 3).




(a) (b)

Figure 3.7. Confocal images of 20% protein dispersions in water at 25 °C for (a) unextruded control and (b) tWPC produced at pH 2.89. The bars represent 10 µm.

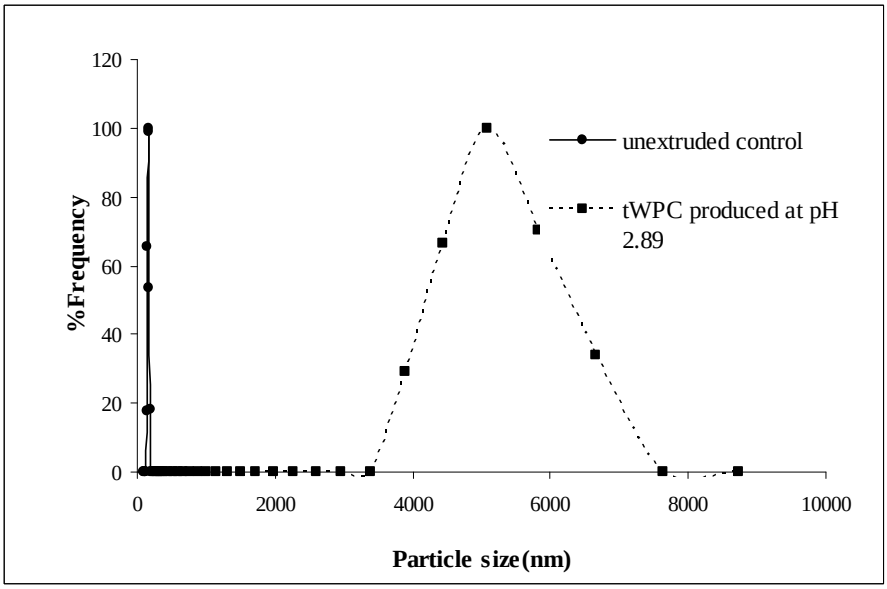



Figure 3.8. Particle size distribution of unextruded control and tWPC produced at pH 2.89. The protein concentration in WP dispersions is 0.1% (w/w). Data are average from 3 tests.

### 3.5. Conclusions

The mechanisms of interactions in WPs during reactive SCFX were highly pH dependent. The disulfide bond formation and the hydrophobic interactions played an important role in the denaturation and aggregation of WPs during reactive SCFX. Generally, the reactive extrusion of WPs resulted in a decrease in protein solubility. Approximately 30% and 80% of proteins in the tWPC produced at pH 2.89 and 8.16, respectively, became insoluble in the standard buffer. The tWPC produced at pH 2.89 was completely soluble in the standard buffer containing urea and SDS. It showed a slight decrease in free SH group contents. This leads to the conclusion that strong protein-protein interactions mainly occurred by non-covalent attractive forces like hydrogen bonds and hydrophobic interactions during reactive SCFX at the acidic pH. This tWPC sample yielded the highest apparent viscosity at equal protein concentrations. At 20% protein concentration, it formed a particulate gel structure and contained particles in the micron-range (mean diameter ~ 5 µm). The tWPC produced at pH 8.16 yielded a grainy texture. It contained a relatively high proportion of large protein aggregates which consequently resulted in a decrease in solubility. These large molecular weight proteins were virtually soluble in the presence of a reducing agent (DTT). It was concluded that the structure of the tWPC produced at pH 8.16 was primarily maintained by intermolecular S-S bonds as evidenced by SDS-PAGE patterns and the dramatic decrease in free SH group content.

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# CHAPTER FOUR

# EMULSIFICATION MECHANISMS AND CHARACTERIZATIONS OF COLD, GEL-LIKE EMULSIONS PRODUCED FROM TEXTURIZED WHEY PROTEIN CONCENTRATE*

## 4.1. Abstract

A novel supercritical fluid extrusion (SCFX) process was used to successfully texturize whey protein concentrate (WPC) into a product with cold-setting gel characteristics that was stable over a wide range of temperature. It was further hypothesized that incorporation of texturized WPC (tWPC) within an aqueous phase could improve emulsion stability and enhance the rheological properties of cold, gel-like emulsions. The emulsifying activity and emulsion stability indices of tWPC and its ability to prevent coalescence of oil in water (o/w) emulsions were evaluated compared with the commercial WPC80. The cold, gel-like emulsions were prepared at different oil fractions ($\varphi$ = 0.20 to 0.80) by mixing oil with the 20% (w/w) tWPC dispersion at 25 ºC and evaluated using a range of rheological techniques. Microscopic structure of cold, gel-like emulsions was also observed by Confocal Laser Scanning Microscope (CLSM). The results revealed that the tWPC showed excellent emulsifying properties compared to the commercial WPC in slowing down emulsion breaking mechanisms such as creaming and coalescence. Very stable with finely dispersed fat droplets, and homogeneous o/w gel-like emulsions could be produced. Steady shear viscosity and complex viscosity were well correlated using the generalized Cox-Merz rule. Emulsions with higher viscosity and elasticity were obtained by raising the oil fraction. Only 4% (w/w) tWPC was needed to emulsify 80% (w/w) oil with long-term storage stability. The emulsion products showed a higher thermal stability upon heating to 85 ºC and could be used as an alternative to

concentrated o/w emulsions and in food formulations containing heat-sensitive ingredients.

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## 4.2. Introduction

Emulsions are thermodynamically unstable systems which tend to be destabilized by several mechanisms including phase inversion, flocculation, aggregation, and coalescence of the dispersed droplets (Dickinson, 1997). Instability of emulsions generally arises if there is insufficient surfactant to cover the entire interface created during homogenization (Dalgleish, 1997). Whey proteins (WPs) have been reported to aid in the formation and stabilization of oil-in-water (o/w) emulsions (Mangino, 1984; Schmidt, Packard, & Morris, 1983) and this functionality is related to their interfacial area and adsorption at the water-oil interface (Dalgleish, 1997; Dickinson, 1997; Pearce & Kinsella, 1978). During emulsification, WP adsorption at water-oil interface is furthered improved due to hydrophobic interactions between a section of the proteins and the oil surface. This creates an interfacial layer which is generally charged (because proteins contain charged amino acids) and can also sterically stabilize the oil droplets (Dalgleish, 1997).

However, surface-active functionality performance of WPs is highly dependent on many factors such as surface hydrophobicity, protein flexibility, heat treatment, pH of the medium, ionic strength, solubility, and protein concentration (Dalgleish, 1997; Fachin & Viotto, 2005; Firebaugh & Daubert, 2005; Pearce & Kinsella, 1978; Voutsinas & Nakai, 1983). Thus, several techniques such as enzymatic, chemical, and physical treatments have been proposed to modify the conformational structure of WPs in order to improve their surface behaviors and emulsifying properties (Dalgleish & Singh, 1998; Demetriades & McClements, 1998; Eynard, Iametti, Relkin, & Bonomi 1992; Fachin & Viotto, 2005; Hunt & Dalgleish, 1994; Kella, Yang, & Kinsella, 1989; Kester & Richardson, 1984; Klemaszewski, Das, Kang, & Kinsella, 1990; Klemaszewski & Kinsella, 1991; Kuehler & Stine, 1974; Monti & Jost, 1978; Nakai, & Li-Chan, 1984; Palazolo, Sorgentini, & Wagner, 2004).

Our previous work has shown that conformational structure and functionalities of whey protein concentrate (WPC) were modified through partial denaturation by means of combined treatments of highly acid treatment (pH<3.0) with heat, shear, and supercritical carbon dioxide (SC-$CO_2$) injection during a supercritical fluid extrusion process (SCFX) in the presence of optimum salt concentrations. Preliminary studies indicated that adding NaCl and $CaCl_2$ at concentration of ~ 64 mM and 33 mM, respectively, to the WP blend could prevent excessive aggregation and promote a stronger structure of protein gels. The texturized WPC (tWPC) was found to possess extremely high viscosity with the ability to form a viscoelastic gel at ambient temperature, whereas the commercial WPC80 always produced a liquid dispersion due to its globular structure (Manoi & Rizvi, 2008). Wagner, Sorgentini, and Añón (1996) indicated that combining acid treatment with heat treatment could improve foaming and emulsifying properties of proteins due to an increase in surface hydrophobicity induced by deamidation and acid-induced denaturation. In general, the conformational modification of proteins as a result of heat treatment leads to partial protein unfolding and generation of additional exposed hydrophobic regions previously buried inside the native structure (Kinsella & Whitehead, 1989; Li-Chan, Nakai, & Wood, 1984). Furthermore, Fachin and Viotto (2005) indicated that the degree of protein conformation and denaturation are considerably more important for emulsifying properties of proteins since they are related to surface hydrophobicity, protein flexibility, stabilizing forces, and a balance of hydrophilic and hydrophobic groups of protein molecules. Britten, Giroux, Jean, and Rodrigue, (1994) also reported that incorporating an optimum level of denatured WPs into an aqueous phase of emulsions increased emulsifying activity, emulsion viscosity, and stability. It was speculated that the whey protein aggregates behave like casein micelle in providing more effective

steric stabilization against coalescence of oil droplets than smaller individual molecules like native WPs or caseinate (Euston & Hirst, 1999).

The complex functions of tWPC such as high solubility, thickening properties, water holding capacity, and surface hydrophobicity have brought the innovative ideas of utilizing this WP derivative product as the polymeric surfactant or emulsifier/stabilizer for food emulsions. In this study, we hypothesized that tWPC could enhance the stability of the emulsion by two major mechanisms; 1) an enhanced adsorption at the oil-water interface which could form a stronger, protective stabilizing layer, and 2) a rheology-modified continuous phase which could better maintain dispersed oil droplet. The improved emulsifying properties of tWPC and its ability to prevent flocculation and coalescence of emulsions could open up new potential uses in food applications.

Moreover, there is considerable interest in converting oil-in-water (o/w) emulsions stabilized by WPs into gels which can be used to create foods with improved organoleptic properties (Sok Line, Remondetto, & Suburade, 2005). These emulsion gels could be produced by inducing gelation of WP-stabilized emulsions by heat treatment (Jost, Baechler, & Masson, 1986; Jost, Dannenberg, & Rosset, 1989). An alternate method of making emulsion gels called ‘cold gelation process’ has been recently reported (Boutin, Giroux, Paquin, & Britten, 2007). Generally, cold emulsion gels are achieved through three consecutive steps: the preparation of pre-heated WP dispersions, the production of o/w emulsions, and the subsequent formation of emulsion gels by the addition of calcium salts (Beaulieu, Savoie, Paquin, & Sabirade, 2002; Sok Line, et al., 2005) or acidification by glucono-δ-lactone (GDL) to pH 4.06 (Boutin et al. , 2007) or 4.80 (Rosa, Sala, Van Vliet, & Van De Velde, 2006).

However, emulsion gels produced by heat treatment have limited uses, especially for food formulations containing heat-sensitive ingredients. The appearance

of emulsion gels prepared by salt-induced gelation is highly dependent on calcium concentrations. Increasing salt concentration produced a particulate gel with poor water holding capacity (Boutin et al., 2007). Additionally, preparation of emulsion gels by salt or acid-induced gelation process is a time consuming process and it is difficult to control the final gel texture. To the best of our knowledge, cold, gel-like emulsions prepared with tWPC, the derivatized WP powder, at ambient temperature have not yet been reported. This approach could be beneficial for controlling the texture of emulsion-filled gel products and their derivatives. In this part of the study, we hypothesized that incorporation of tWPC within an aqueous phase could enhance the rheological properties and thermal stability of o/w emulsions. Our main objectives were to evaluate the emulsification properties of tWPC compared with the commercial WPC80 and to investigate the thermal stability and the effects of oil fractions (20 to 80%, w/w) on the rheological properties of gel-like emulsions.

## 4.3. Materials and methods

### *4.3.1. Materials*

A commercial WPC80 (lactalbumin-493) was obtained from Leprino Foods Company (Lemoore west, CA, USA). The compositions (dry basis) of the commercial WPC80 were 81.5% protein, 5.5% fat, 4% moisture, and less than 3% ash. Corn oil was purchased from a local retailer. Nile red and Fast Green FCF were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA). A powder blend comprising a mixture (by weight) of 94% prehydrated (10% wet basis) WPC80, 6% pre-gelatinized corn starch (Hammond, IN, USA), 0.6% (WPC-starch basis) NaCl, and 0.6% (WPC-starch basis) $CaCl_2$ (Sigma Chemical Co., St. Louis, MO, USA) were preconditioned at ambient temperature overnight before feeding to the extruder. In this process, the pre-gelatinized corn starch has been used as a binder to hold protein

matrices because of their ability to form hydrogen bonds in the extruded product (Amaya-Llano, Morales Hernández, Castaño Tostado, & Martínez-Bustos, 2007). Considerably, the pre-gelatinized corn starch acted as an inactive filler in the tWPC extrudate formation (Manoi & Rizvi, 2008).

### *4.3.2. Production of tWPC by SCFX process*

A pilot-scale Wenger TX-52 Magnum (Wenger Manufacturing, Sabetha, KS, USA) co-rotating twin screw extruder with a length to diameter ratio (L/D) of 28.5 was configured to operate at screw speed of 180 rpm and feed rate of 35 kg/h. The die was fitted with two circular inserts of 1.2 mm diameter each. The die pressure was maintained at 10-15 MPa for continuous SC-$CO_2$ flow into the protein polymer melt, at the desired rate (1% dry feed basis). The 15% (v/v) HCl solution stream was injected into the extruder at the mixing zone to create a pH of about 2.9 and the extrusion was conducted at 60% (dry feed basis) moisture content. The final product temperature was maintained at 90 ºC at the die exit. The extrudate was collected, dried (5-7% moisture content), and grounded using a mill machine (Thomas-Wiley Mill model ED-5, Arthur H. Thomas Co., PA, USA) to reduce the particle size to less than or equal to 1 mm. The tWPC powder was then stored at room temperature in air tight containers until analyzed.

### *4.3.3. Emulsifying properties*

#### *4.3.3.1. Emulsifying activity index and emulsion stability index*

The emulsifying activity index (EAI) and emulsion stability index (ESI) were determined by the turbidometric technique described by Pearce and Kinsella (1978) with some modifications. The emulsions were prepared from 10 mL corn oil and 40 mL of 3% (w/w) tWPC or commercial WPC80 dispersions, adjusted to pH 7. Sodium

azide (0.04% w/w) was added to WP dispersions to prevent microbial growth. Emulsions were then mixed at 25 ºC using a high-speed dispersing and emulsifying unit (model IKA-ULTRA-TURRAX® T25 basic, IKA® Works, Inc., Wilmington, NC) at 21,500 rpm for 2 min. Resulting o/w emulsions (10 μL) were then diluted in 5 mL of 0.1 M phosphate buffer containing 0.1% (w/v) sodium dodecyl sulfate (SDS). The absorbance of the diluted emulsions were then determined in a 1-cm path length cuvette at a wavelength of 500 nm in a Spectronic 1200 spectrophotometer (Bausch and Lomb, Rochester, NY, USA). The turbidity (T) of emulsions was calculated using the following formula:

$$T = \frac{2.303xA}{l} \tag{1}$$

where $A$ is the absorbance at 500 nm, and $l$ is the path length of the cuvette (1 cm).

The emulsifying activity index (EAI) was then calculated as

$$EAI(m^2 g^{-1}) = \frac{2xTxD}{\phi xCx10{,}000} \tag{2}$$

where T is the turbidity, $D$ is the dilution factor, $\Phi$ is the volumetric fraction of oil, $C$ is the weight of protein per unit volume of aqueous phase before the emulsion was formed (g $mL^{-1}$) and 10,000 is the correction factor for square meters. The EAI of emulsions was monitored after storage for 0, 1, and 24 h. The mean of three replicates is reported.

The emulsion stability index (ESI) was calculated after the emulsions were held at 4 ºC for 24 h and reanalyzed for emulsion turbidity as described previously using the following formula:

$$ESI(h) = \frac{(Tx\Delta t)}{\Delta T} \tag{3}$$

where $T$ is the turbidity value at 0 h, $\Delta T$ is the change in turbidity during the storage period, and $\Delta t$ is the time interval. The mean of three replicates is reported.

*4.3.3.2. Creaming index*

Dispersions containing various concentrations of tWPC or commercial WPC80 (0.25, 0.5, 1, 2, 3, and 4%, w/w) in 0.1 M phosphate buffer at pH 7 were prepared at ambient temperature. Sodium azide (0.04% w/w) was added to WP dispersions to prevent microbial growth. Ten millimeters (10 mL) of corn oil and 40 mL of WP dispersion were then mixed at 25 ºC using a high-speed dispersing and emulsifying unit (model IKA-ULTRA-TURRAX® T25 basic, IKA® Works, Inc., Wilmington, NC) at 21,500 rpm for 2 min. The creaming index was evaluated as described by Firebaugh and Daubert (2005) with some modifications. Ten millimeters (10 mL) of each emulsion was filled into a glass test tube (1.5- cm internal diameter x 12-cm height) and then stored at ambient temperature. The height of the serum ($H_s$) and the total height of emulsions ($H_t$) were recorded after storage at ambient temperature for 1, 7, and 14 days. The mean of three replicates is reported. The creaming index was reported as:

$$Creaming\ Index\ (\%) = \frac{H_s}{H_t} x100 \qquad (4)$$

*4.3.4. Cold, gel-like emulsion preparation*

Dispersions containing 20% (w/w) tWPC or commercial WPC80 were prepared in deionized water and stirred for at least 2 h at ambient temperature, and then stored overnight at 4 ºC to ensure complete dissolution. Sodium azide (0.04%, w/w) was added to WP dispersions to prevent microbial growth. Emulsions containing oil levels of 20 to 80 % (w/w) ($\varphi$=0.20, 0.30, 0.40, 0.50, 0.60, 0.70, and 0.80) were

prepared for studying the effect of oil concentrations on emulsion properties. Thus, in this study the protein concentration in aqueous phase was fixed at 20% (w/w). The emulsion of a given oil concentration was prepared by mixing the correct amount of corn oil with the appropriate quantity of aqueous tWPC dispersion, at 9,500 rpm for 3 min using a high-speed dispersing and emulsifying unit (IKA-ULTRA-TURRAX® T25 basic, IKA® Works, Inc., NC, USA). In case of emulsions containing ≥ 60% oil, the pre-emulsion containing 50% oil was first prepared as above. The appropriate amount of oil was then added to the pre-emulsion at the rate of 10 mL/min. The emulsion was continuously mixed while oil was added using a Sunbeam Mixmaster beater at speed 5 until the final emulsion was obtained. The resulting emulsions were then stored in sealed containers at ambient temperature until analyzed.

*4.3.5. Rheological characterization of cold, gel-like emulsions*

The rheological properties of cold, gel-like emulsions were evaluated using a strain-controlled rheometer (ARES, TA Instruments, New Castle, DE, USA) equipped with a Peltier temperature controlling system. A cone and plate geometry (diameter=25 mm, nominal con angle=0.1 radians) was used for steady shear viscosity measurements and the parallel plate geometry (diameter=25 mm, sample thickness=2mm) was used for small-amplitude oscillatory shear experiments. A thin layer of mineral oil was applied to the exposed sample edges to prevent the moisture loss. All measurements were conducted at 25 ºC.

*4.3.5.1. Steady shear viscosities*

Shear rate was ramped from 1 to100 $s^{-1}$. Shear stress, shear rate, and steady shear (apparent) viscosity ($\eta$) were recorded by TA Orchestrator software.

*4.3.5.2. Viscoelastic properties by small-amplitude oscillatory shear (SAOS)*

The frequency was oscillated from 0.1 to 100 rad/s and all measurements were performed within the identified linear viscoelastic region and made at 1% strain. The elastic modulus ($G'$), loss modulus ($G''$), complex viscosity ($\eta^*$), and loss tangent (tan $\delta$) were then recorded by TA Orchestrator software.

*4.3.5.3. Thermal stability*

The temperature was raised from 5 ºC to 85 ºC at 2 ºC/min heating rate and at a constant frequency rate of 1 rad/s and 1% strain. The elastic modulus ($G'$) was then recorded by TA Orchestrator software.

*4.3.6. Confocal microscopy*

The selected emulsion samples were stained with a mixture of Nile Red (0.01%, w/w in a mixture of polyethylene glycol, glycerol, and deionized water (50/45/5)) to visualize the oil phase and Fast Green FCF (0.001%, w/w in deionized water) to visualize the protein phase. The stained emulsion was placed on a glass slide and covered with a cover slide. Confocal laser scanning microscopy (CLSM) was performed on a Leica TCS-SP2 Confocal Laser Scanning head mounted on a Leica DMRE-7 (SDK) upright microscope (Leica Microsystems Inc., Bannockburn, IL, USA) equipped with a 20x HC PL APO/ 0.70NA oil immersion objective lens. 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 green emission range was 500-580 nm and red emission range was 650-730 nm.

*4.3.7. Statistical Analysis*

Statistical analysis was done using MINITAB® release 15 statistical software (State College, PA, USA). Significant differences ($p$<0.05) were determined by analysis of variance using the general linear models and least square means procedure.

## 4.4. Results and discussion

*4.4.1. Emulsifying properties*

The emulsifying activity index (EAI) is related to the surface area stabilized by a unit weight of proteins. It represents the ability of proteins to be adsorbed at the interface of fat globules and the aqueous phase (Pearce & Kinsella, 1978). The results shown in Figure 4.1 reveal that the EAI of commercial WPC80 and tWPC was not significantly different ($p$ < 0.05) at zero-time after emulsion preparation. Graham and Phillips (1980) described that the commercial WPC80 which is mainly composed of native proteins, a highly structured molecule, could be adsorbed at the interface by forming a strong viscoelastic film around the fat globules. On the other hand, the tWPC composed of partially denatured and aggregated proteins as indicated in our previous works possibly achieves the emulsion formation activity by different approach. It is possible that structural changes in tWPC due to denaturation and polymerization induced by reactive SCFX process lead to an increased surface hydrophobicity and molecular flexibility, allowing an effective adsorption of protein molecules at the oil-water interface. It is well documented that denatured proteins usually exhibit a high surface hydrophobicity which enhances emulsifying activity and interfacial concentration by contributing to the film rigidity through hydrophobic interactions between adjacent protein molecules at the interface (Guilmineau & Kulozik, 2007; Kato & Nakai, 1980; Matsudomi, Sasaki, Kato, & Kobayashi, 1985; Mitidieri & Wagner, 2002).

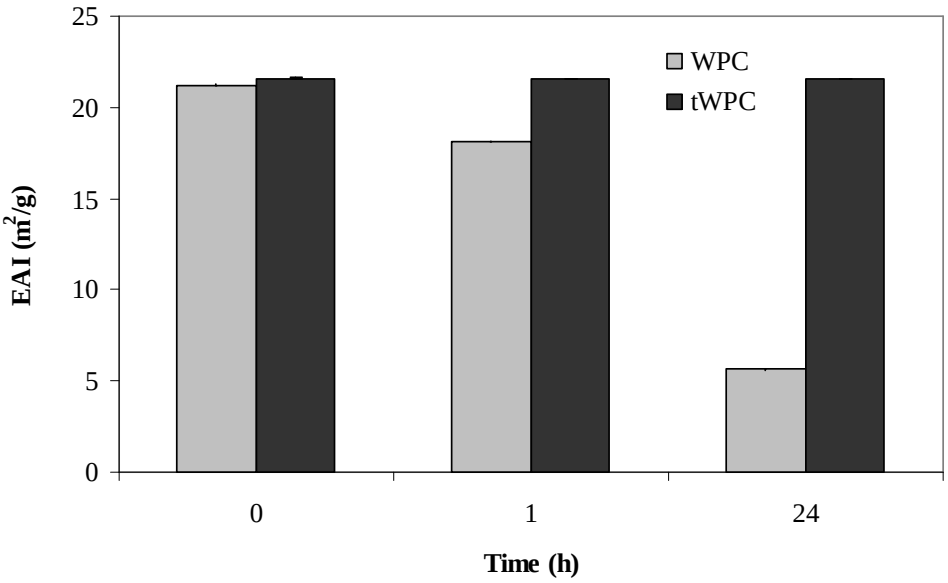



Figure 4.1. Emulsifying activity index (EAI) of commercial WPC80 and tWPC during storage at 25 ˚C for 0, 1, and 24 h. The error bars on data are almost invisible due to small standard deviations.

Interestingly, over longer periods of storage of 1 to 24 h, the EAI of commercial WPC80 was observed to decrease, whereas that of tWPC remained unchanged. The decrease of EAI as a function of time reflects the instability of the commercial WPC80-stabilized emulsion. According to Dalgleish (1997), instability of emulsions occurs when there is either insufficient surfactant to cover the entire oil-water interface or there are gaps in the interfacial layers, thereby decreasing the total adsorbed surface. The emulsion stability index (ESI), on the other hand, reflects the ability of proteins to impart strength to emulsion for resistance against coalescence upon storage (Patel & Kilara, 1990). The greater ESI was observed for tWPC (ESI=13,504 h) compared to that of commercial WPC80 (ESI=33 h), indicating that the emulsion stabilized by tWPC was remarkably more resistant to coalescence. These results imply that the emulsion stability and surface behaviors of native proteins are limited possibly due to their rigid packed globular conformation along with their less molecular flexibility (Kinsella, 1979; Wagner & Guéguen, 1999).

According to the results, the high stability upon storage of diluted emulsions prepared with tWPC is likely due to the formation of a rigid film preventing coalescence of the droplets. This is similar to those reported for some water soluble ampiphilic polymers or macromolecular emulsifiers (Akiyama, Kashimoto, Fukuda, Hotta, Suzuki, & Kitsuki, 2005; Akiyama, Yamamoto, Yago, Hotta, Ihara, & Kitsuki, 2007; Sun, Sun, Wei, Liu, & Zhang, 2007). It is important to note that an essential function of surfactants or emulsifiers is not only that they produce the equilibrium interfacial tension, but also they impart rigidity to the interface by forming films which provide strong repulsive forces between droplets due to a combination of electrostatic and steric interactions, and resistance to rupture (Lucassen-Reynders, 1993; McClements, 1999b). Moreover, viscosity and gelling behaviors of tWPC possibly reflect the hydrodynamic properties of protein macromolecules, in particular,

their shape and size. It has been reported that emulsion flocculation and coalescence could be prevented by inducing a heavily hydrated, charged and a thick interfacial layer (Dagorn-Scaviner, Guéguen, Lefebre, 1987; Graham & Phillips, 1976). These studies also emphasized the importance of the thickness and charge of the protein interfacial layer in preventing coalescence of emulsions. Based on the observations noted above, it is reasonable to speculate that the higher surface activity and better emulsion stability of tWPC compared to the commercial WPC80 could be based on the formation of a thick, rigid film at the oil-water interface through hydrophobic interactions between protein molecules at the interface, and an increase in viscosity of the continuous phase of emulsion.

Emulsion stability could be also observed with respect to creaming and coalescence. In general, coalescence can induce oiling-off and accelerate creaming, which in turn reduces the shelf life of o/w emulsions. The creaming index has been used to indicate the susceptibility of oil droplets to coalescence by such forces as gravitational, colloidal, hydrodynamic, and mechanical, and the resistance of the droplet membrane to rupture during a certain period of time (McClements, 1999b; Pearce & Kinsella, 1978). In this study, the effect of protein concentration on the creaming index was also monitored. As depicted in Figure 4.2, the emulsions stabilized by commercial WPC80 showed separation starting from day 1 of storage following homogenization, whereas the creaming index of tWPC was time and protein concentration dependent. Clearly, the emulsion stability was enhanced by increasing protein concentration. For instance, at lowest protein concentration of 0.25% (w/w), the tWPC-stabilized emulsion was most susceptible to creaming, while the highest protein concentration of 4% was the least susceptible (Figure 4.2).

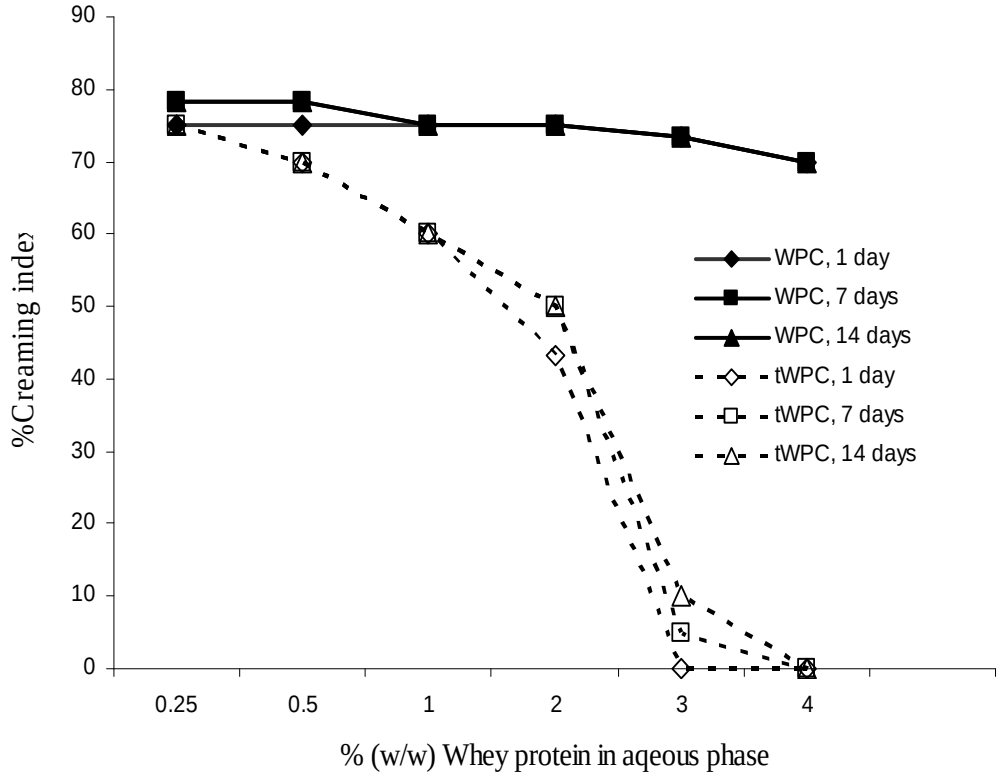



Figure 4.2. % Creaming index of emulsions (20%, w/w oil) stabilized by commercial WPC80 and tWPC at different concentrations in aqueous phase and after storage for 1, 7, and 14 days at ambient temperature. Data are reported as an average of three replications with the maximum error of 5%.

Upon dilution, such emulsions tend to lose the protein stabilization effect. However, at equal protein concentrations, emulsions stabilized by commercial WPC80 coalesced more rapidly than those made with tWPC. The tWPC exhibited lower creaming index at higher protein concentrations. At 2% (w/w) protein concentration, the commercial WPC80 showed nearly twice as much creaming as tWPC. It should be noted that emulsions stabilized with 3% (w/w) tWPC showed little phase separation (creaming index = 7%) even after 14 days of storage at 25 °C, no phase separation was observed in emulsions containing higher protein concentrations (≥ 4%, w/w). Similar results were obtained in emulsions stabilized by derivatized WPs (Firebaugh & Daubert, 2005), protein aggregates (Rosa et al., 2006), and polymeric surfactants (Akiyama et al., 2005, 2007; Sun et al., 2007). Patel and Kilara (1990) and Yamauchi, Shimizu, and Kamiya (1980) indicated that a positive correlation between protein content and emulsion stability was attributed to an increase in the viscosity of the continuous water phase and the amount of protein adsorbed at the fat globule surface. The viscosity of continuous phase is important in predicting the creaming rate of emulsions. According to the Stokes equation (Eq. (5)), the rate of phase separation ($v$) between the continuous phase and dispersed phase depends on densities of the two phases ($\rho_1$ and $\rho_2$), the gravity ($g$), the radius of particles ($r$), and the viscosity ($\eta$) of the continuous phase (Roland, Piel, Delattre, & Evrard, 2003).

$$v = \frac{2r^2(\rho_1 - \rho_2)g}{9\eta} \tag{5}$$

In the previous paper, the tWPC was found to possess remarkably higher viscosity compared to the commercial WPC80 at equal protein concentrations (Manoi & Rizvi, 2008). Furthermore, the thickness of the adsorbed protein layer could be another significant factor in the stability of emulsions. Rosa et al. (2006) reported that

the thickness of adsorbed aggregated protein layer on the droplet surface is higher than that of a native protein by approximately 20 to 26 times.

### *4.4.2. Rheological characterization of cold, gel-like emulsions*

#### *4.4.2.1. Steady shear viscosities*

Our previous work revealed that the tWPC formed a cold-set thickening ability upon reconstitution with water at 20% (w/w) protein concentration (Manoi & Rizvi, 2008). In this study, it was expected that both an associative thickening of tWPC and a protective stabilizing layer on oil droplets would yield stable gel-like emulsions prepared at ambient temperature. Once the oil concentrations in the emulsion system decreased from 80 to 20% (w/w), the final protein and water contents of emulsions would vary from 4 to 16% (w/w), and 16 to 64% (w/w), respectively. Visual observations of emulsions after one day of storage at 25 ºC revealed that all emulsions made from commercial WPC80 remained liquid, while those stabilized by tWPC displayed a self-standing gel with a soft solid-like texture as shown in Figure 4.3.

Changes in viscosities as a function of shear rates of emulsions stabilized by commercial WPC80 and tWPC at various oil mass fractions ($\varphi$=0.20-0.80) are presented in Figure 4.4a and 4.4b, respectively. In general, it showed that at higher oil fractions, emulsions exhibited higher viscosity at a given shear rate. Emulsions prepared with commercial WPC80 exhibited almost Newtonian behavior at oil fractions of 0.20 to 0.60. Figure 4.4a clearly shows the relative viscosity of such emulsions did not change with shear rate. Dimitrova and Leal-Calderon (2004) described that non-flocculated samples generally exhibit a Newtonian flow, while the shear thinning behavior of emulsion is associated with the flocculation of fat droplets. Similar observations were also discussed by Boutin et al. (2007), and Demetriades, Coupland, and McClements (1997).

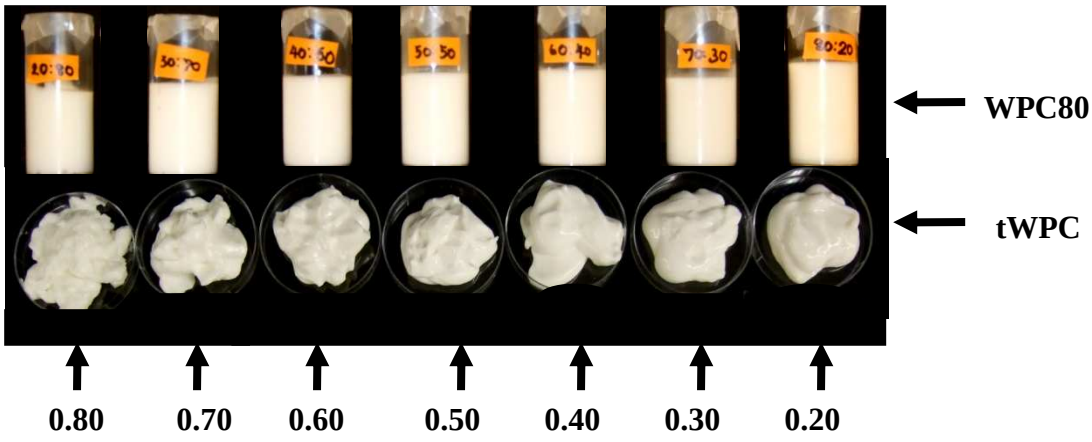
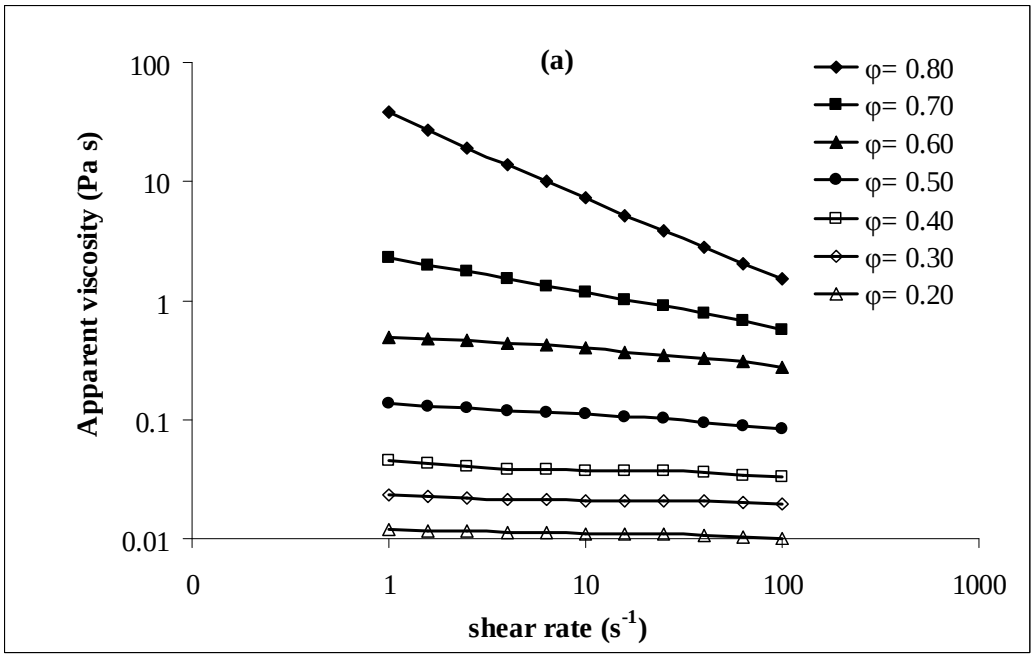



Figure 4.3. Emulsions stabilized by commercial WPC80 (top), and tWPC (bottom) at various oil mass fractions ($\varphi$).

In addition, it was observed that reducing the proportion of oil in commercial WPC80-stabilized emulsions created higher phase separation rate. This is because the interactions between droplets were weakened and emulsions became less stable (Depree & Savage, 2001). However, the commercial WPC80-stabilized emulsions at higher oil fractions of 0.70 and 0.80 exhibited shear-dependent fluids with shear-thinning behavior indicated by the decrease of apparent viscosity with shear rate (Figure 4.4a). It was elucidated that in concentrated emulsions, the droplets were close enough to interact with each other and form the network of aggregated droplets which deformed and consequently disrupted when applied shear rate was increased, resulting in viscosity reduction (Ma & Barbosa-Cánovas, 1995; McClements, 1999a; Liu , Xu, & Guo, 2007). This, they suggested, gives emulsions the higher viscosity due to the flocculation of adjacent oil droplets to form a network. At higher oil concentration, the larger contact surface area between oil droplets opposed the free flow of the emulsion in a shear field, hence increasing its viscosity.

It is interesting to note that when the oil fraction was concentrated up to or above the close packing attainable in a dispersion of monodispse particles ($\varphi^* = 0.64$), the formation of a flocculated emulsion network and retarded coalescence of emulsions was strongly expected (Campanella, Dorward, & Singh, 1995; Dimitrova & Leal-Calderon, 2004; Turgeon, Sanchez, Gauthier, & Paquin, 1996). The authors also mentioned that since the droplets are fluid, they are susceptible to deformation. It means that emulsions can be concentrated up to volume fractions much higher than $\varphi^*$. Similar explanations for the rhelogical behavior of highly concentrated emulsions such as mayonnaise which generally contains about 70-80% (w/w) oil have been pointed



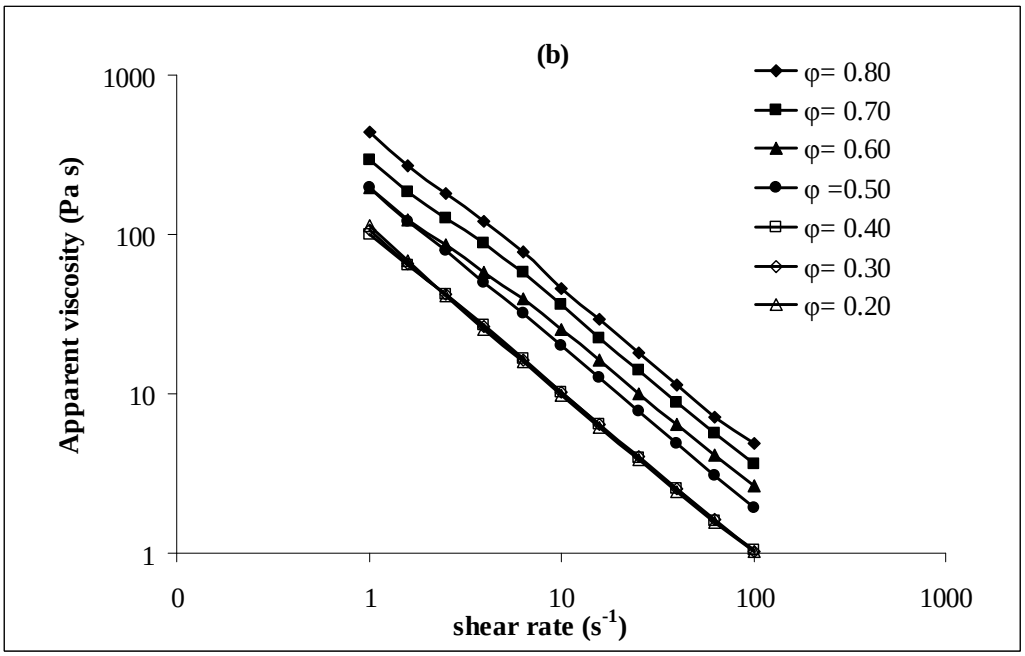




Figure 4.4. Variation of apparent viscosity with shear rate at 25 ºC for emulsions stabilized by (a) commercial WPC80, and (b) tWPC at various oil mass fractions ($\varphi$ = 0.20 to 0.80). The maximum error in data was 5%.

out by Ma and Barbosa-Cánovas (1995). Depree and Savage (2001) stated that the oil may account for 75% or more of the total volume of mayonnaise in which the oil droplets become distorted from their normal, spherical shape, and gives the traditional mayonnaise its high viscosity. However, our emulsion system was also probably close to that for polydisperse spheres. Therefore there may not be enough room for the droplets to move past each other, and so hydrodynamic effects may be as important as the viscosity in controlling stability i.e. it may be close to or above the so-called jamming transition. Bécu, Manneville, and Colin (2006) stated that the effect of inter-particle forces was convincingly demonstrated with moderately concentrated emulsions ($\varphi$ = 0.73), for which jamming transition was observed in the systems with attractive inter-droplet forces only.

Emulsions based on tWPC at all oil concentrations, on the other hand, were very stable upon storage. Apart from its excellent emulsifying properties, tWPC behaved as a gelling agent which could increase the viscosity of the continuous phase. The foregoing results indicated that the apparent viscosity of the 20 % (w/w) tWPC dispersion ($\eta_{6.31}$=7.46 Pa s) was approximately 827 times higher than that of the commercial WPC80 ($\eta_{6.31}$=0.009 Pa s). Thus, the viscosity enhancement of continuous phase is one of the major keys in controlling rheological properties of prepared emulsions. It is believed that the influence of relatively higher viscosity, caused by tWPC in the aqueous phase, resulted in remarkably high emulsion stability even at lower oil contents.

A noticeable shear thinning behavior of emulsions in the presence of tWPC was observed at all oil fractions (Figure 4.4b). The apparent viscosities at the same shear rate of 6.31 $s^{-1}$ ($\eta_{6.31}$) of emulsions based on the commercial WPC80 and tWPC at various oil fractions are compared in Table 4.1. It shows that the apparent viscosities of emulsions with tWPC were significantly ($p < 0.05$) higher than those of

emulsions with commercial WPC80 at comparable oil concentration. In addition, a greater apparent viscosity was also generally observed for emulsions containing higher oil fraction at a given shear rate as shown in Figure 4.4a, 4.4b, and Table 4.1. However, the oil fraction was shown to have less influence on the viscosity for emulsions stabilized by tWPC than in the commercial WPC80. As indicated in Table 4.1, the apparent viscosity ($\eta_{6.31}$) increased by approximately 5 and 1000 times for emulsions stabilized by tWPC and commercial WPC80, respectively, as the oil fraction increased from 0.20 to 0.80. A slight change in viscosity of emulsions based on tWPC with oil fraction may be caused by a counterbalance of the reasonably high viscosity of the continuous phase of emulsions. It was expected that the higher protein content that remained in an aqueous phase participated in the properties of the lamella between the oil droplets, leading to the viscosity enhancement of emulsions.

Results revealed that only 4% (w/w) tWPC (in the final emulsion) was needed to emulsify 80% (w/w) oil. Despite the high oil content relative to water, it was observed that matrix was still o/w emulsion. This indicates the oil droplet entrapment ability, and reduced mobility of droplets and collision frequency of tWPC. Several studies have indicated that the viscosity of the continuous phase of the emulsions and the absorption of the polymers at the oil-water interface are the important keys for the stabilization of the o/w emulsions (Dickinson, 1995; Sun et al., 2007). McClements (2000) reported the thickening agents such as polysaccharides are usually added to o/w food emulsions to enhance the viscosity of the aqueous phase. At sufficiently high concentrations, creaming is retarded because the droplets are incapable of moving in the high viscosity or the gel-network formed by polysaccharides. Nevertheless tWPC was shown to be more advantageous than polysaccharides as an emulsifier by forming an adsorbed layer at the oil-water interface to form a protective steric barrier around droplets, while polysaccharides are usually identified as non-

Table 4.1. Rheological parameters[1] of emulsions stabilized with commercial WPC80 and tWPC at various oil mass fractions.

| Samples/ Oil mass fractions ($\varphi$) | $\eta_{6.31}$[2] (Pa s) | Mechanical spectra[3] | | | |
|---|---|---|---|---|---|
| | | $G'$ (Pa) | $G''$ (Pa) | $\eta^*$ (Pa s) | tan $\delta$ |
| Commercial WPC80 | | | | | |
| 0.80 | 9.95$^{b}$ | 27.90$^{a}$ | 5.37$^{a}$ | 28.41$^{a}$ | 0.19$^{b}$ |
| 0.70 | 1.32$^{a}$ | 5.60$^{a}$ | 3.69$^{a}$ | 6.62$^{a}$ | 0.68$^{a}$ |
| 0.60 | 0.42$^{a}$ | -[4] | - | - | - |
| 0.50 | 0.11$^{a}$ | - | - | - | - |
| 0.40 | 0.04$^{a}$ | - | - | - | - |
| 0.30 | 0.02$^{a}$ | - | - | - | - |
| 0.20 | 0.01$^{a}$ | - | - | - | - |
| tWPC | | | | | |
| 0.80 | 77.78$^{g}$ | 1301.60$^{f}$ | 90.79$^{f}$ | 1304.75$^{f}$ | 0.069$^{f}$ |
| 0.70 | 57.36$^{f}$ | 1001.51$^{e}$ | 70.37$^{d}$ | 1003.98$^{e}$ | 0.070$^{e}$ |
| 0.60 | 39.70$^{e}$ | 894.88$^{d}$ | 77.16$^{e}$ | 898.19$^{d}$ | 0.086$^{d}$ |
| 0.50 | 31.83$^{d}$ | 876.34$^{d}$ | 75.07$^{e}$ | 879.54$^{d}$ | 0.086$^{d}$ |
| 0.40 | 16.65$^{c}$ | 468.29$^{bc}$ | 50.31$^{c}$ | 470.88$^{c}$ | 0.107$^{c}$ |
| 0.30 | 16.37$^{c}$ | 491.59$^{c}$ | 48.45$^{c}$ | 493.97$^{c}$ | 0.099$^{c}$ |
| 0.20 | 15.80$^{c}$ | 415.32$^{b}$ | 40.47$^{b}$ | 417.28$^{b}$ | 0.097$^{c}$ |

[1]Values are the means of three replications, in each column different superscript letters denote significant differences between samples ($p$<0.05).

[2] The apparent viscosity was compared at shear rate of 6.31 $s^{-1}$.

[3] The mechanical spectra were compared at 1 rad/s.

[4] Emulsions were too liquid and unstable to allow dynamic testing.

absorbing colloidal particles (Dalgleish, 2006; McClements, 2000). However, emulsification behaviors of tWPC are believed to be more similar to some polysaccharide derivatives, especially the hydrophobically modified water-soluble polymers based on an associative thickening characteristics and the adsorption of polymers at the interface (Akiyama et al., 2005, 2007; Sun et al., 2007).

*4.4.2.2. Viscoelastic properties*

The oscillation test was performed to establish the relationships between internal structure and flow of emulsions since the viscosity measurement alone was not capable of giving a better understanding of the link between the structure and macroscopic measurable properties. The small strain measurement is believed to leave the microstructure intact and thus makes it possible to characterize the viscoelastic properties of the original sample structure. Emulsions made with commercial WPC80 at oil fractions of 0.20 to 0.60 were not included in viscoelastic results because their very poor consistency prohibited measurement of their dynamic properties in the linear viscoelastic range. In most cases, the loss modulus ($G''$) was higher than the elastic modulus ($G'$) (data not shown) for all frequencies studied, which is a typical behavior of non flocculated or weakly flocculated emulsions, making it impossible to obtain the plateau modulus (Raymundo, Franco, Empis, & Sousa, 2002).

Emulsions made with commercial WPC80 at oil fractions of 0.70 and 0.80 exhibited relative viscous characteristics of a viscoelastic liquid-like material observed from their high frequency-dependence of dynamic moduli (data not shown). For comparison purpose, $G'$, $G''$, $\eta^*$, and tan $\delta$ at frequency of 1 rad/s of emulsions based on commercial WPC80 and tWPC were summarized in Table 4.1. Notably, emulsions stabilized by tWPC investigated here, significantly ($p < 0.05$) yielded higher $G'$, $G''$, and $\eta^*$ than those of commercial WPC80 at comparable oil concentrations. The

elasticity of emulsion gels with tWPC was approximately 178 and 46 times higher than those of emulsions with commercial WPC80 at oil fraction of 0.70 and 0.80, respectively.

Results for tWPC stabilized emulsions showed that dynamic moduli were essentially frequency independent, over the frequency range considered (Figure 4.5). The $G'$ values were approximately one order of magnitude higher than $G''$ values (Figure 4.5 and Table 4.1). It could be interpreted that all emulsion samples behaved in a solid-gel like manner (Clark & Ross-Murphy, 1987). To confirm their gel behaviors, in most of the emulsions studied, a plateau region in the frequency range studied was found. The elastic modulus $G'$ tended to level off when the frequency was very low and close to zero (Figure 4.5), implying that the strong interactions mainly contributing to the elastic modulus needed a long time to relax. At low frequency (long oscillation times), gel-type materials still possessed permanent interactions which gave a predominantly solid behavior. Dickinson and Hong (1995) explained that the development of an entanglement network between adsorbed and non-adsorbed protein molecules is mainly responsible for high elastic modulus and gel-like structure.

Loss factor, tan $\delta$ ($G''/G'$), compares the amount of energy lost to the amount of energy stored indicating whether elastic or viscous properties predominate in a sample. Results in Table 4.1 indicate that the commercial WPC80-stabilized emulsion at oil fraction of 0.80 had tan $\delta$ of 0.19 representing the characteristics of weak predominantly viscous gels, while the emulsion based on oil fraction of 0.70 was rather liquid (tan $\delta$ =0.68). On the other hand, emulsions based on tWPC had tan $\delta$ in a range of 0.07 ($\varphi$=0.80) to 0.10 ($\varphi$=0.20), expressing predominantly strong elastic gels. The variation of tan $\delta$ values with a range of frequencies for emulsions stabilized by tWPC at different oil fractions is presented in Figure 4.6. The slope of tan $\delta$ for most

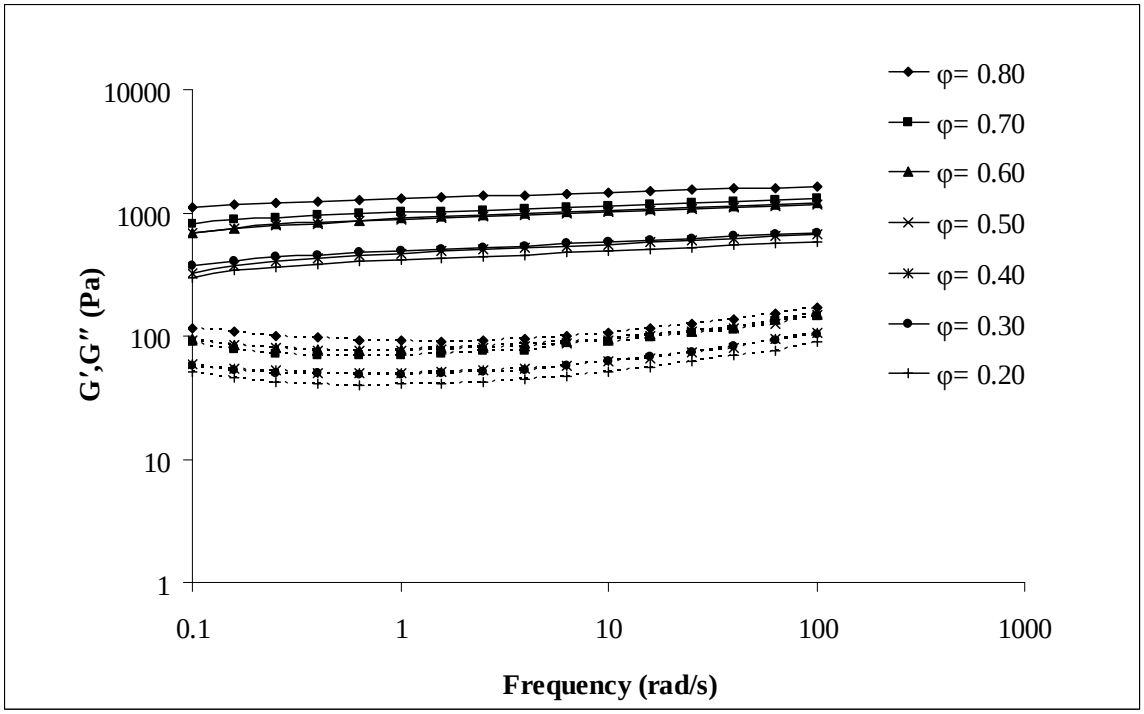



Figure 4.5. Variations of elastic (*G′*, solid lines ) and loss (*G″*, dash lines) moduli with frequency at 25 ºC for emulsions stabilized by tWPC at different oil mass fractions ($\varphi$=0.20 to 0.80). The maximum error in data was 5%.

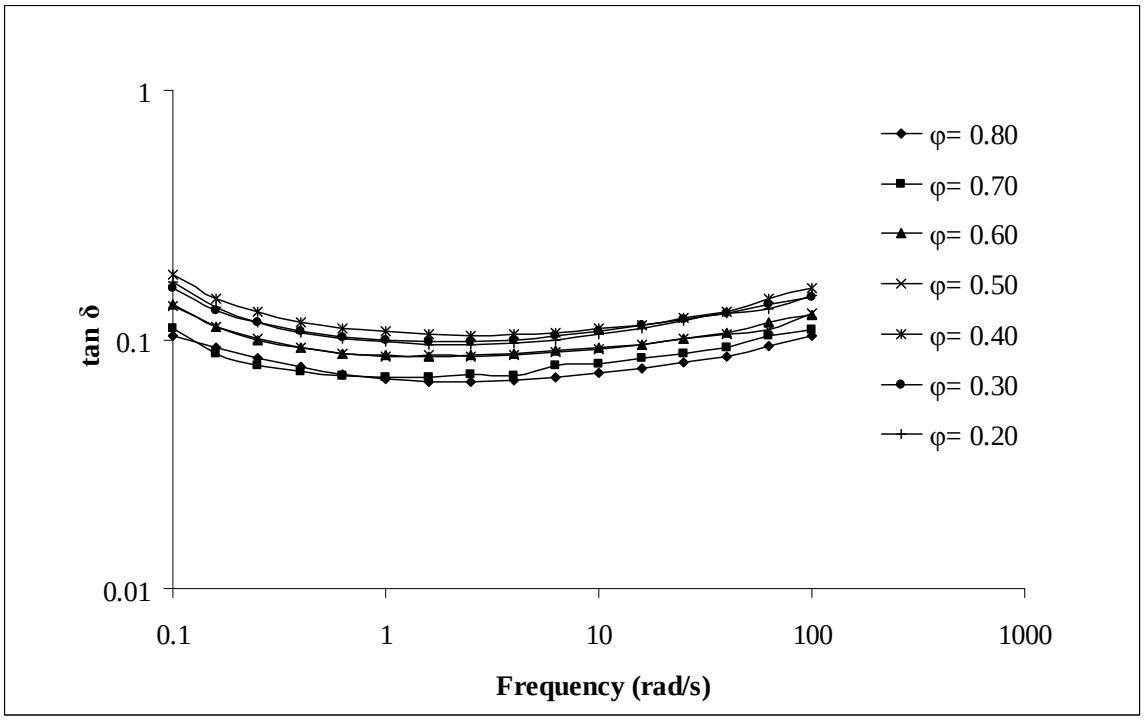
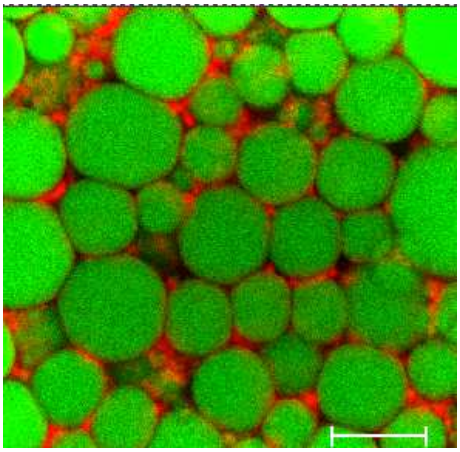



Figure 4.6. Variation of loss tangent (tan $\delta$) with frequency at 25 ºC for emulsions stabilized by tWPC at different oil mass fractions ($\varphi$=0.20 to 0.80). The maximum error in data was 5%.

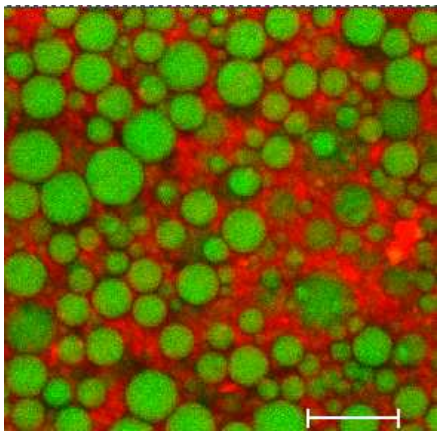
cases was near zero (flat) indicating the frequency independency of the tan $\delta$ which also supported the relatively high elasticity of emulsion gels.

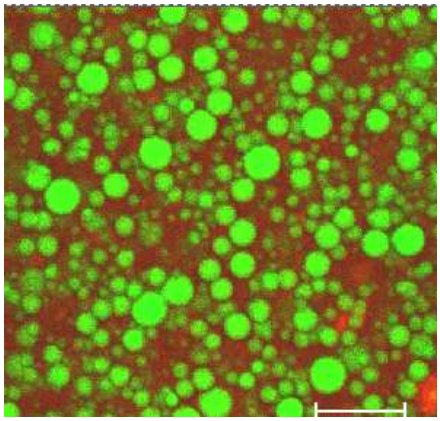
The dynamic mechanical moduli of emulsions showed oil concentration dependence. At higher oil fractions, the noticeably larger $G'$ and $G''$ of both commercial WPC80 and tWPC-stabilized emulsions were shown over an entire range of frequency (Figure 4.5 and Table 4.1). It was also observed that emulsions made with higher oil fractions gave slightly lower tan $\delta$. The increase in $G'$ with oil concentration indicates a more solid, gel-like structure of emulsions. Similar observations have been reported in mayonnaise with xanthan gum (Ma & Barbosa-Cánovas, 1995), highly concentrated protein-stabilized emulsions (Dimitrova & Leal-Calderon, 2004; Hemar & Horne, 2000; Raymundo et al, 2002), heat-set WP-stabilized emulsion gels (Chen & Dickinson, 1998), and cold-set WP-stabilized emulsion gels (Boutin et al., 2007; Rosa et al., 2006; Sok Line et al., 2005). They concluded that at higher oil concentrations, emulsions had more pronounced gel-like characteristics due to more packing and larger size of oil droplets in higher oil concentrations than in the lower oil concentrations.

Microstructure analysis was carried out to determine the distribution of oil droplets in the WP matrix. Figure 4.7 illustrates that oil droplets were homogeneously distributed in the continuous phase of WP gels. These CLSM observations strongly suggest that the fat globule size was larger and closely packed when the oil fraction was higher. The rheological results are well supported by CLSM images observed in selected tWPC stabilized emulsions at three different oil fractions of 0.80, 0.50, and 0.20. The samples that had more compact structure had the higher elastic modulus and lower tan $\delta$. Another possible explanation for the corresponding increase in the elasticity of concentrated emulsions could be droplet repulsion and deformation. As discussed by Dimitrova and Leal-Calderon (2004) above the close packing of



(a)





(b) (c)

Figure 4.7. Confocal laser scanning microscopy (CLSM) images of emulsions stabilized by tWPC at oil mass fraction of (a) 0.80; (b) 0.50; and (c) 0.2. Emulsions were stained with a mixture of Nile red (indicated fat phase, green color) and Fast Green FCF (indicated protein phase, red color). The bar represents 13.5 μm.

equivalent suspension of monodisperse spheres, $\varphi^* = 0.64$, the adjacent droplets forced together will begin to deform before their interface will actually touch due to the repulsive interactions between the droplets, and the emulsions become remarkably rigid and resemble an elastic solid.

Moreover, Chen and Dickinson (1998) pointed out that the dispersed oil droplets can help to build up the gel matrix structure and significantly enhance the gel strength of protein-coated emulsions when they act as the active filler. Many investigations have reported that the oil droplets acted as active fillers, for instance, in heat-set WP –stabilized emulsion gels (Chen & Dickinson, 1998, 1999; Dickinson & Hong, 1995, 1997; Jost et al., 1986; Matsumura, Kang, Sakamoto, Motoki, & Mori, 1993; Yost & Kinsella, 1992), and heat-set modified soy protein isolate-stabilized emulsion gels (Puppo, Sorgentini, & Añón, 2003) due to an increased gel strength with higher oil volume fractions. Based on dynamic mechanical properties of emulsions stabilized by tWPC investigated here, the oil droplets considerably behaved as active fillers and reinforced the gel strength through interactions between adsorbed proteins and those in the gel matrix.

*4.4.2.3. Bohlin's parameters*

The Bohlin's parameters relating $G'$ to frequency were calculated according to the cooperative theory of flow explained by Bohlin (1980) using the following power law model:

$$G' = A\,\omega^{1/z} \qquad (6)$$

where $G'$ is the elastic modulus (Pa), $\omega$ is the frequency (rad/s), and $z$ (dimensionless) and $A$ (Pa) are the power law parameters. According to Bohlin's theory, $z$ is a measure of the extent of the three dimensional network representing the level of these interactions and the coefficient A represents the order of magnitude of the interaction

(Peressini & Sensidoni, 2000; Peressini, Sensidoni, & de Cindio, 1998). The values of *z* and *A* for all emulsions are summarized in Table 4.2. The *z* values ranged from 11.16 to 18.45 for emulsions based on tWPC with 20 to 80% (w/w) oil concentration ($\varphi$=0.20-0.80). From the results, emulsions prepared with 80% (w/w) oil concentration ($\varphi$=0.80) yielded the highest *z* value and coefficient A suggesting the most complex structure and the highest level of interactions between protein-coated droplets. It can be asserted that the compact packing of oil droplets in the protein network is responsible for elastic properties and deformation resistance of the emulsions. According to Peressini et al (1998), the coordination degree between rheological units (*z*) and on interaction strength (*A*) can be correlated with emulsion stability. The authors suggested that low values of *z* and *A* meant the tendency of oil droplets to coalesce when the emulsion undergoes mechanical stress. The results indicated the significantly high stability of all emulsions stabilized by tWPC. It was observed that all emulsions based on tWPC were soft solid-like in texture and very stable with long-term storage ($\geq$ 6 months). In contrast, the oil release was visible in the majority of emulsions stabilized by commercial WPC80 after a short-time of storage. These findings are clearly related to emulsification activities of both proteins and their ability to stabilize emulsions as discussed in the first part of this work.

*4.4.3. Applicability of Cox-Merz rule between steady shear and oscillation*

The empirical Cox-Merz rule states that the magnitude of the complex viscosity ($\eta^*$) and the steady shear viscosity ($\eta$) must be superimposed at equal values of frequency and shear rate as presented by Eq. (7) (Cox & Merz, 1958). An important feature of this rule is the establishment of a correlation between large deformations, the steady shear flows, which are basically non-linear and the small and linear deformations, the small-amplitude oscillatory shears (SAOS) (Gunasekaran & Ak,

Table 4.2. Power law parameters[a] ($A$ and $z$) of emulsions stabilized with commercial WPC80 and tWPC at various oil mass fractions.

| Samples/Oil mass fractions ($\varphi$) | Power law parameters | | |
|---|---|---|---|
| | $A$ (Pa) | $z$ | $R^2$ |
| Commercial WPC[b] | | | |
| 0.80 | 28.08 | 6.52 | 0.97 |
| 0.70 | 5.33 | 2.61 | 0.99 |
| tWPC | | | |
| 0.80 | 1286.40 | 18.45 | 0.99 |
| 0.70 | 982.53 | 16.29 | 0.98 |
| 0.60 | 872.82 | 13.79 | 0.97 |
| 0.50 | 857.02 | 14.10 | 0.98 |
| 0.40 | 451.49 | 11.16 | 0.96 |
| 0.30 | 478.85 | 12.22 | 0.98 |
| 0.20 | 401.56 | 11.78 | 0.97 |

[a]Values are the means of three replications; $A$-the proportional coefficient; $z$-the coordination number ( $G' = A\,\omega^{1/z}$ ).

[b]Emulsions at $\varphi$ = 0.20 to 0.60 were too liquid and unstable to allow dynamic testing.

2000). A relationship between the steady shear and oscillatory data is valuable to correlate true material properties obtained from different tests (Gunasekaran & Ak, 2000; Steffe, 1996).

$$\eta^* = \eta\big|_{\omega=\dot{\gamma}} \tag{7}$$

The power law parameters relating steady shear viscosity to shear rate and complex viscosity to frequency for tWPC-stabilized emulsions at various oil fractions are presented in Table 4.3. It shows that values of *A* were approximately 3 times less than values of *B* in all cases. The $\eta^*$ and $\eta$ values of three emulsion samples ($\varphi$=0.20, 0.50, and 0.80) are presented in Figure 4.8 as functions of frequency and shear rate. It illustrates that the Cox-Merz rule did not fit $\eta^*$ and $\eta$ plotted at equivalent frequencies (0.1-100 rad/s) and shear rates (0.1-100 $s^{-1}$) for all tWPC-based emulsions. Parallel dependencies of $\eta^*$ on frequency and $\eta$ on shear rate were obtained with the values of $\eta^*$ higher than the $\eta$ values from continuous shear ramps (Figure 4.8). It means that samples did not hold the Cox–Merz rule. However, the result showed that the commercial WPC80-stabilized emulsion based on 80% (w/w) held the Cox-Merz rule over a shear rate/frequency range of 0.1-10 $s^{-1}$(data not shown). Departures from this rule have been reported to occur in structured polymer systems such as in polymer liquid crystals or when aggregation takes place among polymer chains and highly-branched starch (Chamberlain & Rao, 2000; Lapasin & Pricl, 1995). Gunasekaran and Ak (2000) described that departures from Cox-Merz rule are attributed to structural decay due to the extensive strain applied. Though applied strain is low in SAOS, it is sufficient enough in steady shear to break down structured inter and intra-molecular associations of materials (Ahmed & Ramaswamy, 2006; Gunasekaran & Ak, 2000). According to some authors (Rao, Okechukwu, Da Silva, & Oliveira, 1997; Tárrega, Durán, & Costell, 2005), deviations from the Cox-Merz rule may imply strong inter and intra-molecular associations or a gel-like structure.

Table 4.3. Power law parameters for steady shear viscosity ($\eta$) versus shear rate ($\dot{\gamma}$) and complex viscosity ($\eta^*$) versus frequency ($\omega$) and modified Cox-Merz rule parameters for emulsions stabilized with tWPC.

| Oil mass fraction ($\varphi$) | Steady shear ($\eta = A\dot{\gamma}^a$) | | Oscillatory shear ($\eta^* = B\omega^b$) | | Mod. Cox-Merz ($\eta^* = K\eta^\alpha$) | |
|---|---|---|---|---|---|---|
| | $A$ | $a$ | $B$ | $b$ | $K$ | $\alpha$ |
| 0.80 | 447.99 | 0.986 | 1290.60 | 0.946 | 3.76 | 0.957 |
| 0.70 | 307.33 | 0.955 | 985.89 | 0.939 | 3.55 | 0.982 |
| 0.60 | 205.11 | 0.935 | 877.28 | 0.928 | 4.47 | 0.992 |
| 0.50 | 198.37 | 1.004 | 861.33 | 0.929 | 6.45 | 0.925 |
| 0.40 | 103.54 | 1.004 | 455.14 | 0.911 | 6.76 | 0.907 |
| 0.30 | 105.06 | 1.008 | 482.13 | 0.918 | 6.96 | 0.910 |
| 0.20 | 107.01 | 1.024 | 404.36 | 0.915 | 6.20 | 0.894 |

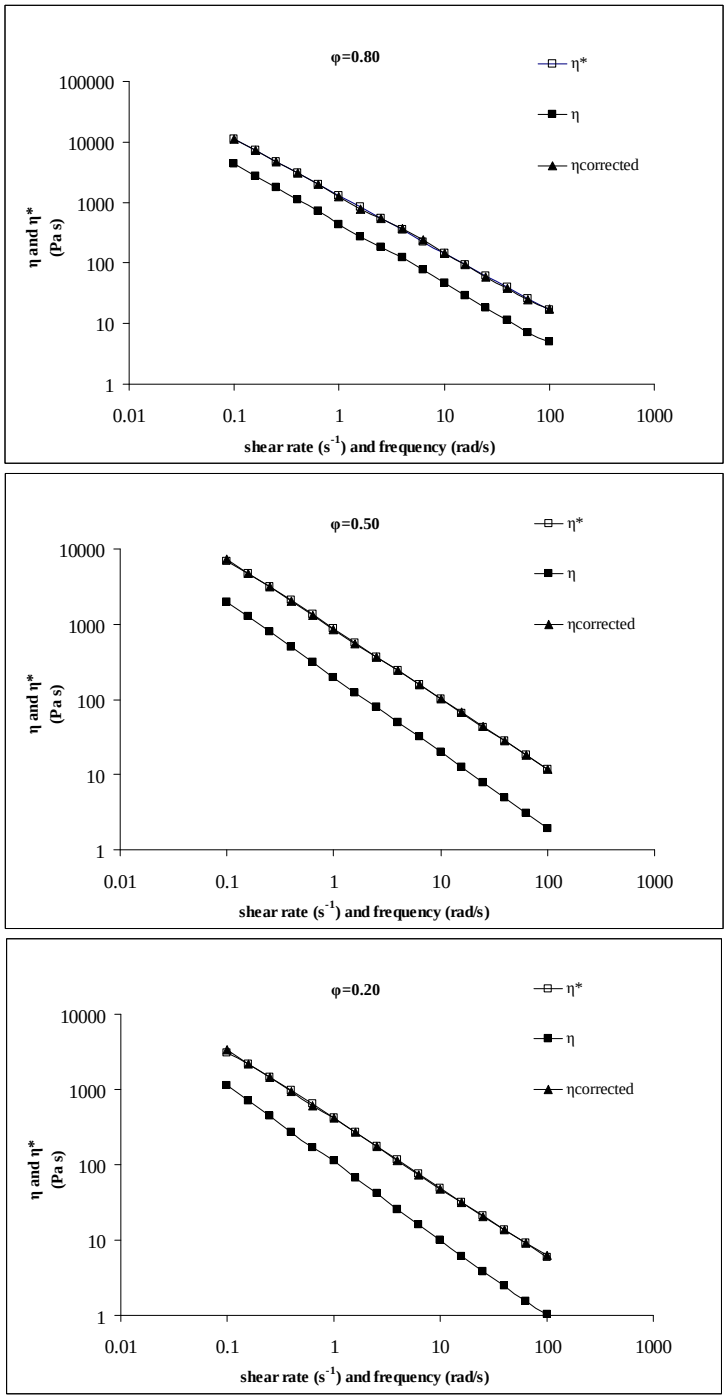



Figure 4.8. Cox-Merz correlation between oscillatory and steady shear responses for emulsions stabilized by tWPC at selected oil mass fractions of 0.80, 0.50, and 0.20 at 25 ºC.

Although, the Cox-Merz rule proved inadequate for studied emulsions, the modified version of this rule with the introduction of constant $K$ (shift factor) and $\alpha$ values supplied a useful relationship (Eq. (8)). This is called generalized Cox-Merz rule (Gunasekaran & Ak, 2000). The suitability of the generalized Cox-Merz relation was studied by fitting both the variations of $\eta^*$ and $\eta$ data with frequency/shear rate to the power law. Results revealed that the $\eta^*$ and $\eta$ data were represented well by the generalized Cox-Merz relation ($R^2 > 0.999$). The power law parameters ($K$ and $\alpha$) for emulsions based on tWPC are presented in Table 4.3.

$$\eta^* = K\eta^{\alpha}\Big|_{\omega=\dot{\gamma}} \tag{8}$$

The parameters, $K$ and $\alpha$, obtained from Eq. (8) can be implemented to compute a 'corrected' steady shear viscosity ($\eta_{corrected}$). The applicability of generalized Cox-Merz for studied emulsions is shown by the $\eta_{corrected}$ values that match the complex viscosity over a shear rate/frequency range studied as presented in Figure 4.8. A generalized Cox-Merz relation was also observed to hold for several semi-solid food materials such as apple butter, mustard, cream cheese, margarine, sweet potato puree infant food, derivatized WP gels, and diary desserts (Ahmed & Ramaswamy, 2006; Bistany & Kokini, 1983; Resch, Daubert, & Foegeding, 2004; Tárrega et al., 2005; Yu & Gunasekaran, 2001).

*4.4.4. Thermal stability of cold, gel-like emulsions*

Results showed that emulsions stabilized by tWPC were far less sensitive to heat treatment than emulsions based on commercial WPC80. All emulsions based on tWPC displayed little variation in elastic modulus ($G'$) when the temperature was raised from 5 ºC to 85 ºC (Figure 4.9). A slight variation in $G'$ of these emulsions after the temperature ramping could be attributed to the additional unfolding and

denaturation of native WPs remaining in an aqueous phase. In previous work, our results showed that the SCFX process rendered WPC into a tWPC powder with cold-setting gel characteristics that was stable during heat treatment. A similar trend was expected for this study and all emulsions stabilized by tWPC were relatively stable over a wide range of temperature. On the other hand, most emulsions prepared with commercial WPC80 partially coagulated and exhibited a hard texture and phase separation after emulsions were heated to 85 ºC (data not shown). This is obviously related to denaturation of the native WPs mainly in the commercial WPC80 sample.

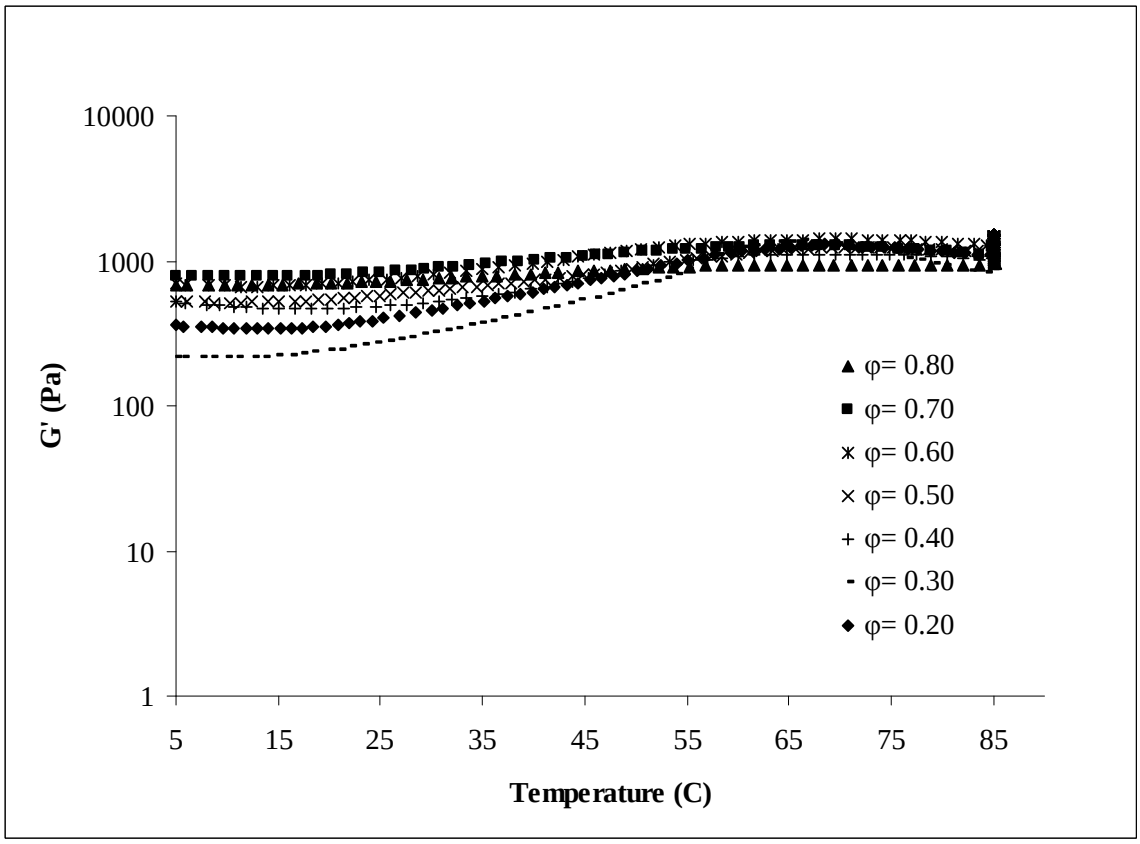



Figure 4.9. Variations of elastic modulus (*G'*) with temperature for emulsions stabilized by tWPC at different oil mass fractions ($\varphi$=0.20 to 0.80). The maximum error in data was 5%.

## 4.5. Conclusions

This study demonstrates that tWPC exhibited excellent emulsifying properties. It may be suggested that there are two possible stabilization mechanisms in the emulsion prepared with tWPC. First mechanism serves for emulsifying capability and the second mechanism serves for the stability effect, preventing creaming. These mechanisms, alone or synergistically, were responsible for the higher surface activity and emulsion stability of tWPC compared to that of commercial WPC80.

The homogeneous gel-like emulsions with various oil concentrations could be successfully produced at ambient temperature by incorporation of tWPC within an aqueous phase. Oil in water, gel-like emulsions based on tWPC were very stable and had finely dispersed fat droplets. Emulsions with higher apparent viscosity and elasticity were obtained by raising the oil concentration since oil droplets acted as active filler and strengthened the emulsions. The gel-like emulsions at all oil concentrations demonstrated markedly thermal stability indicated by little variation in rheological properties upon heating to 85ºC. The results of this study confirmed our hypothesis that reactive SCFX rendered WPC into an ingredient with excellent gelling and emulsifying properties. This may open up a new avenue for utilization of tWPC in food emulsions, especially in food formulations containing heat-sensitive ingredients.

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# CHAPTER FIVE

# STABILITY AND RHEOLOGICAL PROPERTIES OF CORN OIL AND BUTTER OIL EMULSIONS STABILIZED WITH TEXTURIZED WHEY PROTEIN CONCENTRATE

## 5.1. Abstract

The influence of texturized whey protein concentrate (tWPC) produced by supercritical fluid extrusion, on the stability of oil-in-water type emulsions was studied. Emulsions of butter oil as well as corn oil were prepared at different oil concentrations (20, 50, and 80% w/w) by mixing liquid oil with 20% (w/w) tWPC dispersion in water and stored at different temperatures (5, 25, and 55 °C) for up to 7 days. Emulsion morphology was observed by Confocal Laser Scanning Microscopy (CLSM) and quantified for size distribution of oil droplets while rheological behavior was examined to establish their stability. In all cases, a homogeneous, gel-like emulsion of creamy consistency was produced when liquid oil was emulsified in the continuous aqueous phase containing tWPC. During storage, all corn oil-based emulsions were stable against droplet coalescence, and no free liquid oil was observed. However, the stability of butter oil-based emulsions was significantly influenced by storage temperatures and oil concentrations. All butter oil emulsions were stable when stored at 55 °C. Microscopy provided further evidence of droplet coalescence in 50 and 80% butter oil emulsions during quiescent storage at 5 and 25 °C. Emulsions with higher elasticity were obtained by raising the oil fraction. The extent of fat solidification during low temperature storage significantly increased the elasticity of emulsions made of butter oil.

## 5.2. Introduction

In oil-in-water (o/w) emulsions, the oil is dispersed as liquid droplets through the continuous phase, which is usually but not necessarily water. Such emulsions are thermodynamically unstable due to several mechanisms including phase inversion, creaming, flocculation, aggregation, and coalescence of the dispersed droplets (Dickinson, 1997). To prevent destabilization, the boundary separating the dispersed and continuous phases generally contains a surface-active component such as proteins, surfactants, and phospholipids (McClements, 1999). However, the oil is less dense than the aqueous phase and thus the dispersed droplets are prone to floating upwards and accumulating at the surface. This process is called creaming and can be prevented by the addition of stabilizers which increase the viscosity of the continuous phase. A polysaccharide like xanthan gum is often used as a stabilizer in emulsion products like soups, sauces, and dressings due to its excellent ability to increase the aqueous phase viscosity. McClements (2000) stated that at sufficiently high concentrations of polysaccharides in aqueous phase, creaming is retarded because the droplets are incapable of moving in the high viscosity or the gel-network of the continuous phase. However, most polysaccharides are usually identified as non-absorbing colloidal particles (Dalgleish, 2006; McClements, 2000). On the other hand, emulsifying agents like proteins reside at the oil-water interface and help preserve emulsion stability (Coia & Stauffer, 1987; Nor Hayati, Che Man, Ping Tan, & Nor Aini, 2009). Therefore, a combination of protein and polysaccharide is often employed in order to increase the emulsion stability by providing a high viscosity as well as a charged thick, gel-like absorbed surface active layer at the system interface (Sun, Gunasekaran, & Richard, 2007; Tolstoguzov, 1997).

The stability of protein-stabilized emulsions depends on several factors including protein adsorbed layer structure, viscosity of the continuous phase, emulsion

aggregation and rheology, storage temperature, protein concentration, average droplet size, droplet size distribution, oil volume fraction, type of oil, and fat crystallization (Dalgleish, 1997; Friberg, Goubran, & Kayali, 1990; Lizarraga, Pan, Añon, & Santiago, 2008; Rousseau, 2000; Sun & Gunasekaran, 2009). The important properties of emulsions are their stability against creaming, droplet coalescence, and their rheological behaviors. Stability against creaming and coalescence relate to shelf life of emulsion products. The rheological behavior of emulsions is extremely important for the choice of formulation, texture and sensory characteristics, consumer perception, process conditions, and quality control. In food emulsions, the magnitude of the oil volume fraction has profound effects on the rheological properties of the emulsion. The volume fraction of oil can range from a few percent like in homogenized milk to greater than 80% as in mayonnaise. Such versatility can be exploited to produce a very large set of products from flowing liquids with Newtonian viscosities to elastic, solid-like materials (Hemar & Horne, 2000).

In our previous study we reported the production of texturized WPC (tWPC) by a supercritical fluid extrusion process (SCFX) and its rheological properties (Manoi & Rizvi, 2008). It was observed to possess extremely high viscosity with the ability to form a viscoelastic gel at ambient temperature. In addition, our preliminary study showed that due to its enhanced surface-activity and increased surface hydrophobicity, tWPC can be easily absorbed at the oil-water interface and protect against creaming. In this study, we reported on how droplet coalescence could be reduced by incorporating tWPC in an aqueous phase of o/w emulsions. This could be achieved through the dual role played by tWPC involving enhancement of viscosity of the aqueous phase and adsorption at the oil-water interface. Higher viscosity helps minimize the mobility of oil droplets and subsequently retards the collision frequency and reduces the droplet coalescence in o/w emulsions. The emulsification behaviors of

tWPC have similarities to surface-active polysaccharides such as guar gum, gum arabic, modified starch, cellulose derivatives, and locust bean gum, and polysaccharide derivatives such as hydrophobically modified water-soluble polymers based on an associative thickening characteristics and the adsorption of polymers at the interface (Akiyama, Kashimoto, Fukuda, Hotta, Suzuki, & Kitsuki, 2005; Akiyama, Yamamoto, Yago, Hotta, Ihara, & Kitsuki, 2007; Dickinson, 2003; Nor Hayati et al., 2009; Sun, Sun, Wei, Liu, & Zhang, 2007).

It is well known that emulsions are susceptible to destabilization and partial coalescence when fat crystals are formed in the dispersed phase upon cooling (Boode & Walstra, 1993; Relkin, Sourdet, & Fosseux, 2003; Rousseau, 2000). Van Boekel and Walstra (1981) proposed that partial coalescence may be caused by fat crystals located at the oil-water interface. Partial coalescence occurs when two or more partially crystalline emulsion droplets come into contact. When two droplets approach each other, a fat crystal protruding from the droplet may pierce the thin film layer of adsorbed protein between closed droplets, which leads to conjunction of the droplets, resulting in the formation of an irregularly-shape aggregate (Van Boekel & Walstra, 1981). Upon reheating, the fat crystal melts and the partially coalesced droplets collapse and merge together, allowing full coalescence and oiling off. Thus, the aim of the present study was to incorporate tWPC into an aqueous phase of emulsions and to examine their stability against coalescence. The effect of storage temperatures and oil concentrations (20 to 80% w/w) on the microstructure and rheological properties of emulsions containing butter oil (crystallizable oil) as well as corn oil was taken into consideration.

## 5.3. Materials and Methods

### *5.3.1. Materials*

Commercial WPC-80 was obtained from Leprino Foods Company (Lemoore west, CA, USA). Unsalted sweet cream butter and corn oil were purchased from a local retailer. All other reagents were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA).

### *5.3.2. Anhydrous butter oil preparation*

Butter was melted at 60 ºC and centrifuged at 3000 g for 5 min to separate the butter oil from protein and other materials. The top butter oil layer was decanted and stored at -20 ºC until needed.

### *5.3.3. Production of tWPC by SCFX process*

A feed formulation comprising (w/w) 94% prehydrated (10% wet basis) WPC-80, 6% pre-gelatinized corn starch (Hammond, IN, USA), 0.6% NaCl, and 0.6% $CaCl_2$ was texturized using a reactive SCFX. A pilot-scale Wenger TX-52 Magnum (Wenger Manufacturing, Sabetha, KS, USA) co-rotating twin screw extruder with a length to diameter ratio (L/D) of 28.5 was configured to operate at screw speed of 180 rpm and feed rate of 35 kg/h. The average specific mechanical energy (SME) input for the process was 57 Wh/kg. The die was fitted with two circular inserts of 1.2 mm diameter each. The die pressure was maintained at 10-15 MPa for continuous SC-$CO_2$ flow into the protein polymer melt, at the desired rate (1% dry feed basis). A solution of 15% (v/v) HCl was injected into the extruder at the mixing zone to obtain pH of about 2.9. Extrusion was conducted at 60% (dry feed basis) moisture content. The final product temperature was maintained at 90 ºC at the die exit. The extrudate was collected, dried at ambient temperature (final moisture content ~ 5-7%) and ground using a mill

machine (Thomas-Wiley Mill model ED-5, Arthur H. Thomas Co., PA, USA) to reduce the particle size to less than or equal to 1 mm. The tWPC powder was then stored at room temperature in air tight containers.

*5.3.4. Emulsion preparation*

A 20% (w/w) tWPC dispersion was prepared by reconstituting tWPC powder in deionized water and allowed to stir for at least 2 h at ambient temperature. It was then stored overnight at 5 ºC to ensure complete dissolution. Sodium azide (0.04%, w/w) was added to the dispersion to prevent microbial growth. Emulsions containing oil levels of 20, 50, and 80 % (w/w) were prepared for studying the effect of oil concentrations on emulsion properties. The emulsion of a given oil concentration was prepared by mixing the correct amount of corn oil as well as butter oil (maintained at 55 ºC) with the appropriate quantity of aqueous tWPC dispersion, at 9,500 rpm for 3 min using a high-speed dispersing and emulsifying unit (IKA-ULTRA-TURRAX® T25 basic, IKA® Works, Inc., NC, USA). In case of 80% (w/w) oil emulsions, the pre-emulsion containing 50% oil (w/w) content was first prepared as above. The appropriate amount of oil was then added to the pre-emulsion at the rate of 10 mL/min. The emulsion was continuously mixed while oil was added using a Sunbeam Mixmaster beater at speed 5 until the final emulsion was obtained. Emulsions with butter oil were prepared at 55 ºC to assure that fat was completely melted, while those with corn oil were prepared at ambient temperatures. The resulting emulsions were then stored in sealed containers under three different conditions: refrigerated storage at 5 ºC; ambient storage at 25 ºC; and at 55 ºC in an oven.

*5.3.5. Emulsion stability by centrifugation assay*

In the first part of the investigation, the stability of freshly prepared and stored (7 days at 5, 25, and 55 ºC) emulsions was evaluated by centrifugation assay. Emulsions were accurately weighted (10 g) into a Teflon centrifuge tube, then heated at 40 ºC for 15 min, and centrifuged at 21,000 g for 15 min at 35 ºC using a Sorvall® Evolution RC Super-speed Centrifuge (Thermo Electron Corporation, NC, USA). The height of an aqueous phase, an emulsion phase, and oil phase on top of emulsions after centrifugation was recorded. The emulsion stability (ES) was calculated as a percentage: ES (%) = (the height of the emulsion phase/ the total height of emulsion) x 100. All samples were assayed in triplicate.

*5.3.6. Droplet size distribution*

The droplet size distribution of emulsions and was evaluated immediately after preparation and after 7 days of storage at different temperatures using a laser diffraction method of Mastersizer 2000 (Malvern Instruments Ltd., UK). The droplet size distribution was determined based on the best fit between the experimental measurements and the Mie theory (McClements, 2005). Emulsions were diluted (0.5 % w/w) with warmed (~40 ºC) 1% sodium dodecyl sulfate (SDS) solution and stirred gently for 20 min prior to measurement. The sample solution was then centrifuged at 6000 g for 15 min at 35 ºC (Sorvall® Evolution RC Super-speed Centrifuge, Thermo Electron Corporation, NC, USA) in order to separate precipitates such as whey protein aggregates which could create multiple scattering effects. The emulsion solution was carefully decanted and re-dispersed in warmed 1% SDS solution. Drops of emulsion solution were introduced into the sample presentation unit and dispersed in deionized water at 1200 rpm and 40 ºC until an obscuration rate of about 8% was obtained. Optical properties of the sample were defined as follow: a refractive index (RI) of

1.458, a dispersant RI of 1.33 (water), and absorption 0.00 to calculate the Dispersion Index (Span) by *Span* = *d*[90]-*d*[10]/*d*[50]. The *d*[90], *d*[50], and *d*[90] values are size values corresponding to the cumulative distribution at 10%, 50%, and 90% (Palazolo, Sorgentini, & Wagner, 2004). Droplet size was reported as the volume-weighted mean diameter: $d_{4,3} = \Sigma n_i d_i^4 / = \Sigma n_i d_i^3$, where $n_i$ is the number of droplets of diameter $d_i$. The measurements were done in triplicate.

*5.3.7. Confocal microscopy*

The selected emulsion samples were stained with a mixture of Nile Red (0.01%, w/w in a mixture of polyethylene glycol, glycerol, and deionized water) to visualize the oil phase and Fast Green FCF (0.001%, w/w in deionized water) to visualize the protein phase. The stained emulsion was placed on a glass slide and covered with a cover slide. Confocal laser scanning microscopy (CLSM) was performed on a Leica TCS-SP2 Confocal Laser Scanning head mounted on a Leica DMRE-7 (SDK) upright microscope (Leica Microsystems Inc., Bannockburn, IL, USA) equipped with a 20x HC PL APO/ 0.70NA oil immersion objective lens. 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 green emission range was 500-580 nm and red emission range was 650-730 nm.

*5.3.8. Viscoelastic properties of emulsions by small-amplitude oscillatory shear (SAOS)*

The rheological properties of emulsions were evaluated using a strain-controlled rheometer (ARES, TA Instruments, New Castle, DE, USA) with a cone and plate (diameter = 25 mm, nominal cone angle = 0.1 radians) geometry. Temperature was controlled by a Peltier system located in the base of the measurement geometry. A

thin layer of mineral oil was applied to the exposed sample edges to prevent the moisture loss. The frequency was oscillated from 0.1 to 10 rad/s and all measurements were performed within the identified linear viscoelastic region and made at 1% strain. The elastic modulus ($G'$), loss modulus ($G''$), and complex viscosity ($\eta^*$) were then recorded by TA Orchestrator software. The measurement for fresh emulsions was carried out within 1 h after preparation at 25 ºC while the analysis for stored emulsions was carried out at their storage temperature.

## 5.4. Results and discussion

### *5.4.1. Emulsion stability (ES)*

The stability of emulsions after storage at 5, 25, and 55 ºC for 7 days in comparison with the freshly prepared sample was observed with respect to coalescence and phase separation upon reheating at 40 °C. The stability of emulsions was determined at 40 ºC in which the crystalline fat was completely melted. Coalescence is indicative of emulsion destabilization, where two or more droplets coagulate and form larger droplets or oiling off. The droplets thus coalesce into progressively larger masses of oil, which in extreme cases may result in a continuous oil phase separated from aqueous phase by a single interface. It was observed that almost no oil was separated from the freshly prepared emulsions in all cases upon reheating at 40 ºC, suggesting that tWPC was capable of protecting the emulsified liquid oil. This agrees with the findings obtained in our previous studies showing that tWPC exhibited excellent emulsifying properties compared to the commercial WPC-80 in slowing down emulsion breaking mechanisms such as creaming and droplet coalescence (Manoi & Rizvi, 2009). The outcome of previous studies suggested that there were two possible stabilization mechanisms in the emulsion prepared with tWPC. The first mechanism is the adsorption of the tWPC at the oil-water interface

which prevented coalescence between the droplets. The second mechanism is an increase in viscosity of the continuous phase which prevented creaming by trapping the oil droplets within the gel matrix. These mechanisms, alone or synergistically, were responsible for the higher surface activity and emulsion stability of tWPC compared to that of commercial WPC-80. Moreover, the results showed that there was no significant effect of storage temperature and oil concentration on the stability of emulsions containing liquid oil. Data for all emulsions prepared with corn oil were not included since they were stable from coalescence over a storage period of 7 days, and no large oil droplets or free running oil were observed at the top of emulsions (ES=100%). In addition, emulsions with corn oil showed a strong resistance to serum and oil separation upon reheating at 40 ºC.

On the other hand, the stability of emulsions with butter oil was obviously influenced by storage temperature and oil concentration. Figure 5.1 indicates that all 20% butter oil emulsions were stable and showed no oiling off over the storage period (ES=100%). In 50% butter oil emulsions, oiling off was observed after storage at 5 ºC and reheating. The emulsion stability of this sample was reduced by 43%, whereas emulsions stored at 25 and 55 ºC were stable over a period of 7 days. Clearly, there was a trend towards decreased emulsion stability with increasing oil content during lower temperature storage. Results shown in Figure 5.1 confirm that the destabilization was more extensive in emulsions containing 80% butter oil during chilling storage. It is interesting to note that the overall appearance of the 80% butter oil emulsion was retained at 5 ºC, while the free oil was observed at 25 ºC. It is possible, however, that partial coalescence may have occurred on a microscopic scale. Upon reheating at 40 ºC, the severe oil separation under centrifugation force was observed in 80% butter oil emulsions after storage at 5 and 25 ºC for 7 days. The 80% butter oil emulsion was least stable at 5 ºC (ES=18 %), followed by at 25 ºC

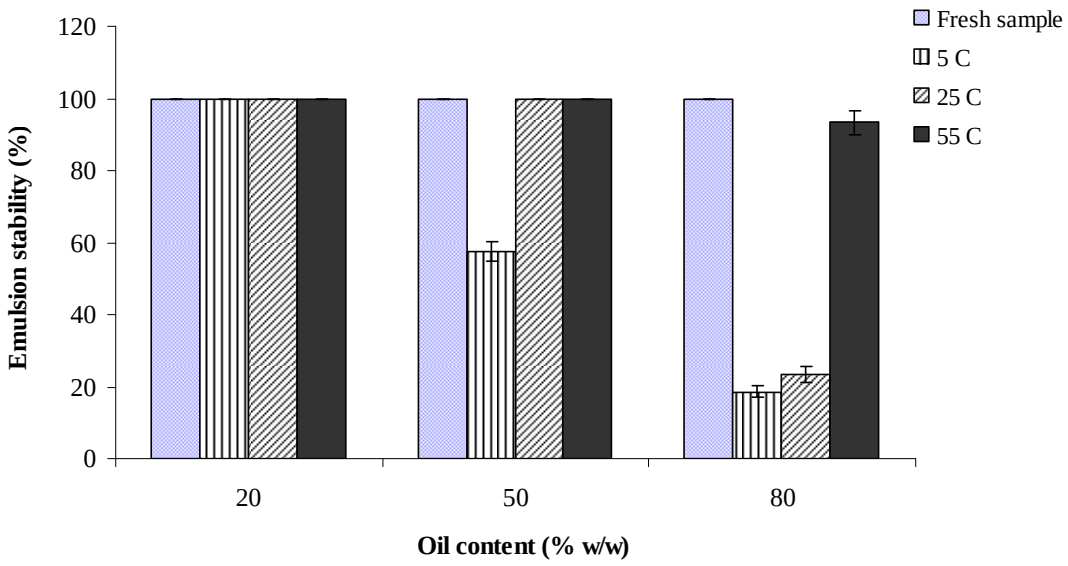



Figure 5.1. Emulsion stability (%) of freshly prepared and stored (5, 25, and 55 °C, 7 days) emulsions based on butter oil at various oil contents. Error bars represent standard deviation from the average of 3 replications.

(ES=23%). This observation implied that at lower temperature the partial coalescence was strong enough to cause full coalescence and finally oiling off after the fat crystal melted upon reheating at 40 ºC. It is the fact that milk fat is semicrystalline over a wide temperature range and unstable during temperature changes (Rousseau, 2000). Kiokias, Reiffers-Magnani, and Bot (2004) reported that during quiescent cooling the overall o/w character of the emulsion was retained. However, during the temperature cycling, recrystallization of emulsified fat formed the more thermodynamically stable larger crystals that may invert the emulsion upon reheating.

The significant impact of storage temperature on the destabilization of emulsions containing butter oil was expected especially at higher oil concentration. For the emulsions made of crystallizable oil, it was reported that besides emulsifier type, lipid composition including solid fat content, fat crystal and shape plays a vital role in destabilization of the emulsion during storage and temperature cycling (Kiokias et al., 2004; Vanapalli, Palanuwech, & Coupland, 2002). Darling and Birkett (1987), Rousseau (2000), and Van Boekel and Walstra (1981) also reviewed the role of fat crystallization in the destabilization of o/w emulsions. The authors described that partial coalescence in o/w emulsions required some fat crystals to be protruding from the droplet surface. In addition, crystallization induction in other droplets can be caused by crystal penetration into neighboring droplets and acting as nucleation sites for subsequent growth. Our previous studies have shown that with increasing oil concentration, the fat droplets were packed more closely and the inter-particle distance is reduced (Manoi & Rizvi, 2009). It is probable that fat crystals pierced the thin film (adsorbed protein layer at the oil-water interface) between droplets, and may therefore favor a partial coalescence as soon as a crystal from one droplet touched the oil phase of another. Additionally, the crystal is wetted better by the oil than water, and with time the droplets fuse more closely to reduce the surface area of the oil exposed to the

aqueous phase (Cramp, Docking, Ghosh, & Coupland, 2004; Davies, Dickinson, & Bee, 2000; Van Boekel & Walstra, 1981). The fat crystals melted upon reheating above the final melting point of butter fat (~37 ºC), allowing coalescence and oil separation. However, the result showed that all emulsions were quite stable during storage at 55 ºC. This is because the emulsion droplets were completely liquid at 55 ºC and behaved similarly to the emulsified corn oil. Although some stored emulsion samples were susceptible to oiling off upon reheating, it was observed that all emulsions showed a strong resistance to serum separation (syneresis) after centrifugation. This is due to the excellent water holding capacity of tWPC as well as its ability to enhance the emulsion viscosity which retarded synerisis.

*5.4.2. Droplet size distribution*

Emulsion stability can be evaluated with respect to changes in droplet size distribution through time. In the present study the droplet size distribution of emulsions was characterized immediately after emulsions were taken out of storage conditions. Droplet size in the range of 0.02 to 2000 μm was detected by the particle size analyzer. It appears that the droplet size distribution of all emulsions with corn oil was found to be mono-modal (Figure 5.2a, c, e). Table 5.1 shows the mean diameters of cumulative droplet distributions, dispersion index, and volume-weight mean particle diameter ($d_{4,3}$) of corn oil emulsions. In case of freshly prepared emulsions, the volume-weight mean particle diameter ($d_{4,3}$) was in the narrow range of 3.77 to 6.55 μm with oil content variation (20 to 80%, w/w), suggesting that the droplet size was slightly influenced by the amount of oil in the emulsions. However, the emulsion with 80% corn oil showed the lowest degree of polydispersity (*Span* = 1.13) among the freshly prepared samples, indicating good uniformity of the droplets.

**Corn oil emulsions** **Butter oil emulsions**

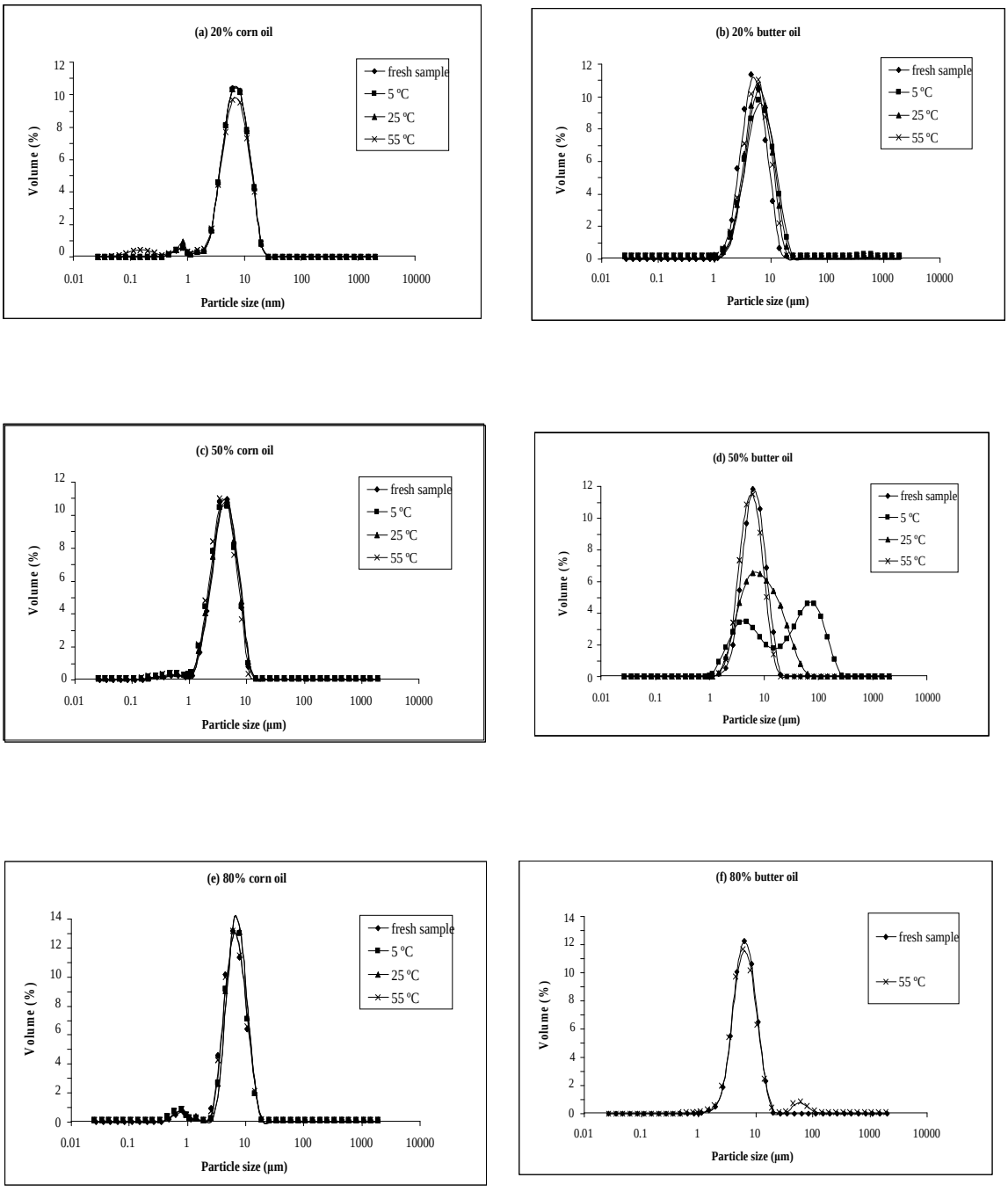



Figure 5.2. Droplet size distribution of freshly prepared and stored (5, 25, and 55 °C, 7 days) emulsions prepared with corn oil containing 20 (a), 50 (c), and 80% (e) oil content and with butter oil containing 20 (b), 50 (d), and 80% (f) oil content.

Table 5.1. Droplet mean diameters[a] (μm) of freshly prepared and stored emulsions based on various concentrations of corn oil.

| Oil contents (% w/w) | Storage temperature | Corn oil | | | | |
|---|---|---|---|---|---|---|
| | | *d*[10] μm | *d*[50] μm | *d*[90] μm | *Span* | *d*[4,3] μm |
| 20 | Fresh | 2.922 | 5.982 | 11.223 | 1.388 | 6.549 |
| | 5 ºC | 2.930 | 6.009 | 11.325 | 1.397 | 6.590 |
| | 25 ºC | 2.916 | 5.989 | 11.274 | 1.395 | 6.565 |
| | 55 ºC | 2.184 | 5.748 | 11.163 | 1.661 | 6.240 |
| | Stdv. range | 0.003-0.009 | 0.001-0.008 | 0.001-0.021 | 0.001-0.011 | 0.001-0.011 |
| 50 | Fresh | 1.731 | 3.473 | 6.333 | 1.325 | 3.770 |
| | 5 ºC | 1.642 | 3.429 | 6.411 | 1.391 | 3.747 |
| | 25 ºC | 1.731 | 3.541 | 6.519 | 1.352 | 3.851 |
| | 55 ºC | 1.609 | 3.289 | 5.984 | 1.330 | 3.555 |
| | Stdv. range | 0.0005-0.004 | 0.0005-0.003 | 0.0005-0.015 | 0.0005-0.001 | 0.0005-0.004 |
| 80 | Fresh | 3.164 | 5.597 | 9.476 | 1.128 | 5.951 |
| | 5 ºC | 3.542 | 5.932 | 9.555 | 1.014 | 6.206 |
| | 25 ºC | 3.567 | 5.969 | 9.609 | 1.012 | 6.244 |
| | 55 ºC | 3.197 | 5.661 | 9.583 | 1.128 | 6.009 |
| | Stdv. range | 0.0005-0.004 | 0.001-0.003 | 0.002-0.006 | 0.0005-0.001 | 0.001-0.004 |

[a] Data are reported as an average of three replications for each sample.

The droplet distribution of prepared emulsions was well demonstrated by their corresponding droplet morphology observed under the microscope (Figure 5.3a, c, and e). The CLSM images illustrate that oil droplets were homogeneously distributed in the continuous phase of tWPC gel matrix. These CLSM observations strongly suggest that the fat globule size was closely packed when the oil fraction was higher. The droplet size for corn oil emulsions was not significantly influenced by storage temperature and this evidence was supplemented by the CLSM results (data not shown). This observation suggests that emulsions were stable with respect to droplet size during storage and correlates very well with the foregoing emulsion stability results. As shown in Figure 5.2a, c, and e the size distributions were still mono-modal and not apparently changed with storage temperatures.

Both the size distributions and the average droplet sizes of freshly prepared emulsions with butter oil were comparable to those of emulsions based on corn oil since the emulsified oil remained liquid during the tests. The size distributions for freshly prepared butter oil emulsions were all mono-modal and were slightly different between the emulsions (Figure 5.2b, d, and f). All fresh butter oil emulsions had a volume-weight mean particle diameter ($d_{4,3}$) ranging from 4.77 to 6.18 μm. The droplet morphology for freshly prepared emulsions with various oil concentrations was displayed in Figure 5.3b, d, and f.

As expected, considerable changes in both droplet sizes and distributions with storage temperatures were observed in butter oil emulsions. As shown in Table 5.2 and Figure 5.2b, d, and f, most changes in droplet size occurred at 5 ºC, while minimal changes of droplets were exhibited at 55 ºC for all oil concentrations. The volume-weight mean particle diameter ($d_{4,3}$) of the 20% and 50% butter oil emulsion shifted from 4.77 μm (fresh sample) to 9.45 μm , and from 6.18 μm (fresh sample) to 38.63 μm, respectively, after storage at 5 ºC for 7 days (Table 5.2). During storage at

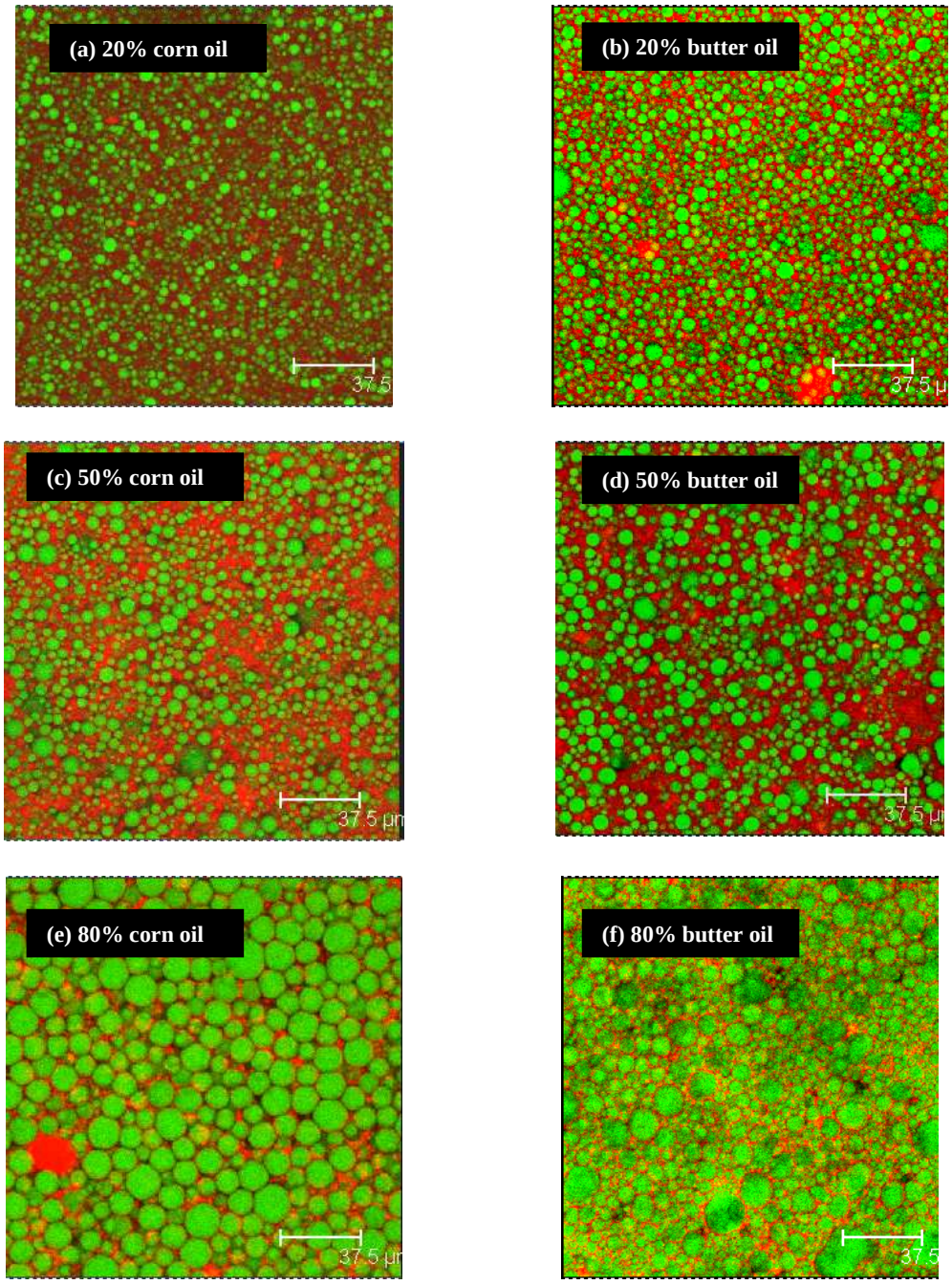



Figure 5.3. Confocal laser scanning microscopy (CLSM) images of freshly prepared emulsions prepared with corn oil containing 20 (a), 50 (c), and 80% (e) oil content and with butter oil containing 20 (b), 50 (d), and 80% (f) oil content. Emulsions were stained with a mixture of Nile red (indicated fat phase, green color) and Fast Green FCF (indicated protein phase, red color). The bar represents 37.5 μm.

Table 5.2. Droplet mean diameters[a] (μm) of freshly prepared and stored emulsions based on various concentrations of butter oil.

| Oil contents (% w/w) | Storage temperatures | Butter oil | | | | |
|---|---|---|---|---|---|---|
| | | *d*[10] μm | *d*[50] μm | *d*[90] μm | *Span* | *d*[4,3] μm |
| 20 | Fresh | 2.264 | 4.316 | 7.966 | 1.321 | 4.767 |
| | 5 ºC | 2.657 | 5.602 | 11.435 | 1.567 | 9.445 |
| | 25 ºC | 2.728 | 5.416 | 10.466 | 1.429 | 6.085 |
| | 55 ºC | 2.582 | 5.074 | 9.445 | 1.352 | 5.601 |
| | Stdv. range | 0.003-0.048 | 0.0005-0.397 | 0.007-0.522 | 0.0005-0.002 | 0.005-0.014 |
| 50 | Fresh | 3.099 | 5.655 | 10.189 | 1.236 | 6.179 |
| | 5 ºC | 2.477 | 23.094 | 99.951 | 4.221 | 38.629 |
| | 25 ºC | 2.799 | 7.372 | 21.718 | 2.566 | 10.201 |
| | 55 ºC | 2.703 | 4.982 | 8.970 | 1.258 | 5.464 |
| | Stdv. range | 0.0005-0.025 | 0.003-1.376 | 0.007-3.654 | 0.0005-0.003 | 0.003-1.462 |
| 80 | Fresh | 3.103 | 5.560 | 9.754 | 1.196 | 6.045 |
| | 5 ºC | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 25 ºC | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 55 ºC | 3.127 | 5.712 | 11.001 | 1.378 | 8.090 |
| | Stdv. range | 0.001-0.002 | 0.003-0.028 | 0.010-0.033 | 0.0005-0.002 | 0.004-0.128 |

[a] Data are reported as an average of three replications for each sample.

n.d.: Not detected.

room temperature (~ 25 ºC), results showed that the droplet size still increased, but to a much lesser extent than at 5 ºC , from 4.77 μm (fresh sample) to 6.09 μm for the 20% butter oil emulsion, and from 6.18 μm (fresh sample) to 10.20 μm for the 50% butter oil emulsion, respectively. Overall, the 20% butter oil emulsions were stable with storage conditions with respect to droplet size and morphology (CLSM results were not included).

Notably, storing the 50% butter oil emulsion at 55 ºC did not significantly influence the droplet morphology (Figure 5.4a) and it was identical to the fresh sample (Figure 5.3d). Although the emulsion did not show oil separation on visual inspection, relative changes in droplet size distribution (Figure 5.2d) and morphology (Figure 5.4c) were seen in this sample during storage at 25 ºC for 7 days. The result shown in Table 5.2 indicates that the degree of polydispersity (*Span* = 2.57) of this sample and the mean diameter below which 90% of the volume of the droplet ($d[90]$ = 21.72 μm) increased more than two times. The larger value of $d[90]$ could further confirm the presence of new larger droplets. It is generally known that an increase in the polydispersity and droplet mean diameter upon storage is a clear indicator of droplet coalescence. The microstructure of the 50% butter oil emulsion stored at 25 ºC demonstrates the presence of many larger droplets and several droplets with irregular non-spherical shape (Figure 5.4c). The formation of non-spherical droplets could be attributed to partial crystallization of fat crystals upon cooling (Boode & Walstra, 1993). It is quite possible that the 50% butter oil emulsion has a tendency to coalesce with prolonged storage time at room temperature if the fat crystals are substantially enlarged and protrude through a thin film of absorbed proteins at the interface. However, the 50% butter oil emulsion stored at 25 ºC for 7 days did not show oil separation upon reheating as previously discussed. This could be because the tWPC matrix gel was strong enough to hold the partially crystallized fat and retarded the

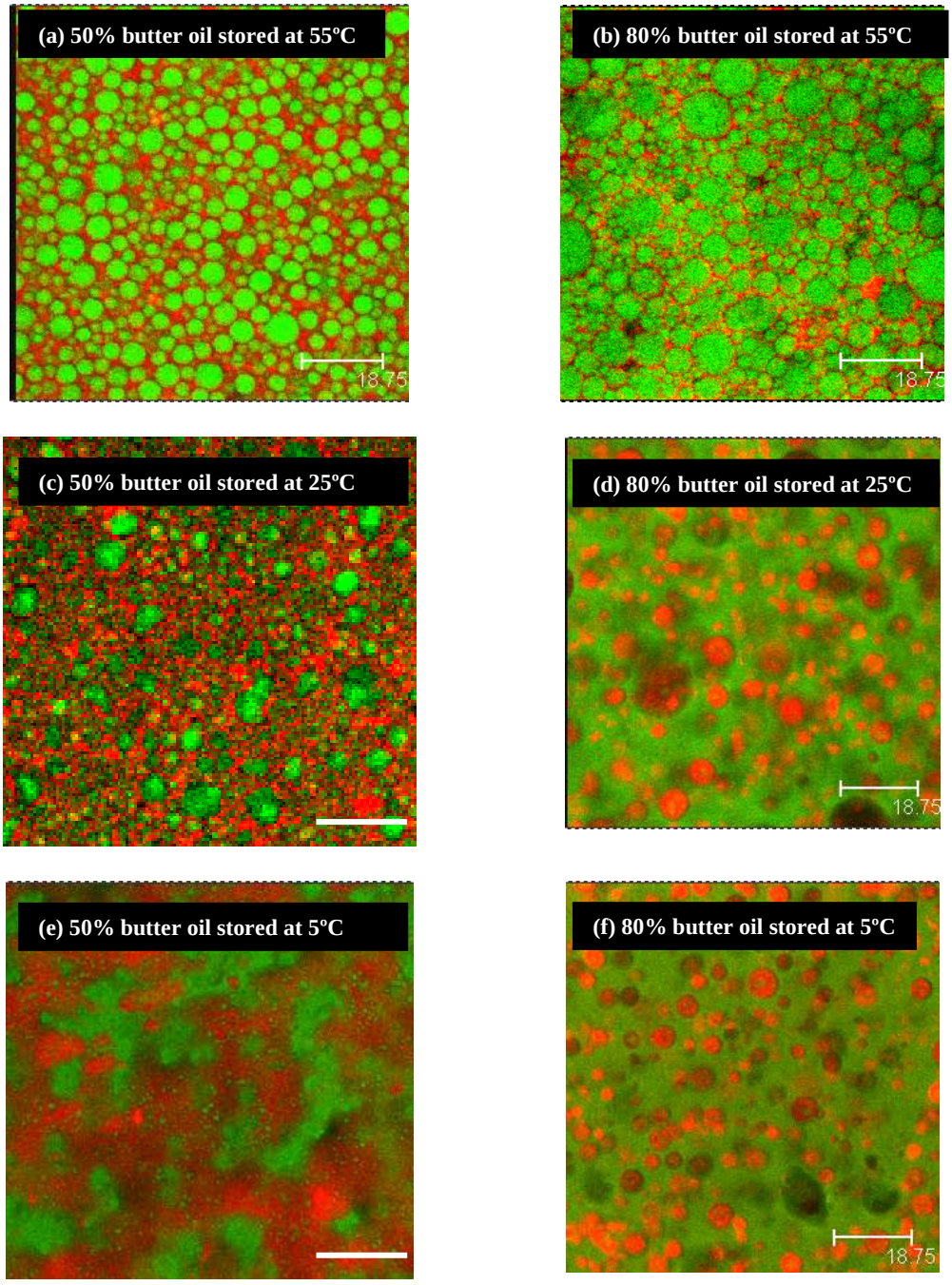



Figure 5.4. Confocal laser scanning microscopy (CLSM) images of emulsions prepared with 50% butter oil stored at 55 °C (a), 25 °C (c), and 5 °C (e) and emulsions prepared with 80% butter oil stored at 55 °C (b), 25 °C (d), and 5 °C (f) for 7 days.

Emulsions were stained with a mixture of Nile red (indicated fat phase, green color) and Fast Green FCF (indicated protein phase, red color). The bar represents 18.75 μm.

droplets to coalesce when the emulsion was exposed to higher temperature. Another explanation could be that at room temperature the fraction of crystallized fat within the droplets was not sufficient to cause the partial coalescence.

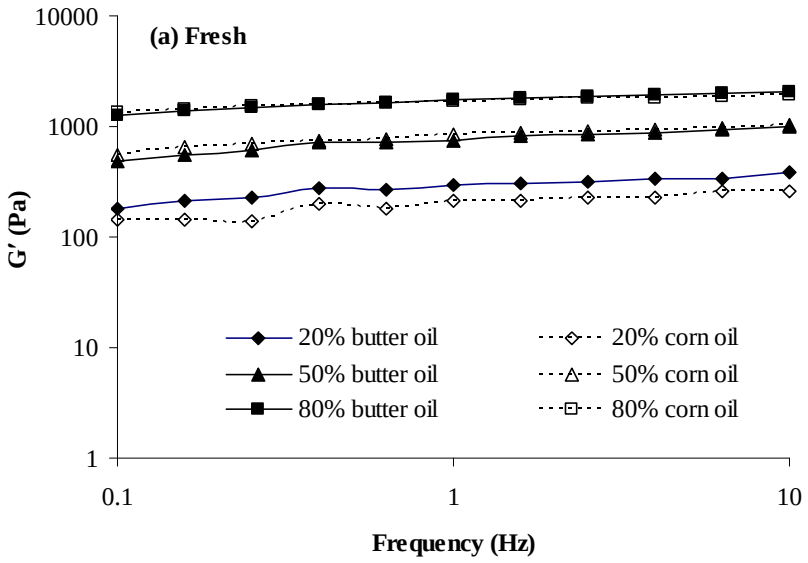
The rise in larger droplets of the 50% butter oil emulsion is more pronounced during storage at 5 ºC. As a result, the polydispersity degree of this emulsion significantly increased by almost four times (*Span* = 4.22). The substantial increase in the median mean diameter ($d[50]$ = 23.10 μm) and the mean diameter below which 90% of the volume of the droplet ($d[90]$ = 99.95 μm) existed was also revealed in this emulsion (Table 5.2). Meanwhile, the mean diameter below which the minority group of the droplets existed (10% of the cumulative distribution) was similar to the others (Table 5.2). Figure 5.2d demonstrates that the droplet distribution of the 50% butter oil emulsion stored at 5 ºC transformed into a bimodal, consisting of a minor peak corresponding to a fraction of small droplets and a major peak corresponding to a fraction of new large droplets. A shoulder of a major peak is attributed to new large droplets with > 100 μm. This is in agreement with the CLSM results. The more severe droplet coalescence of the 50% butter oil emulsion occurred at 5 ºC as shown in Figure 5.4e. It indicates that upon reheating to higher temperature the liquid oil flowed out to preferentially wet the fat crystals and reinforced the contact point, causing the droplet coalescence. According to the results, storing the 50% butter oil emulsion in the refrigerator accelerated the droplet coalescence. According to Davies et al. (2000), droplet coalescence is maximized when the solid fat content is in the approximate range of 10 to 50%. It is important to note that the anhydrous milk fat is expected to contain approximately 50 % solid fat at 5 ºC and less than 10% solid fat at 25 ºC (Kaylegian & Lindsay, 1992). Therefore, we expected a more extensive

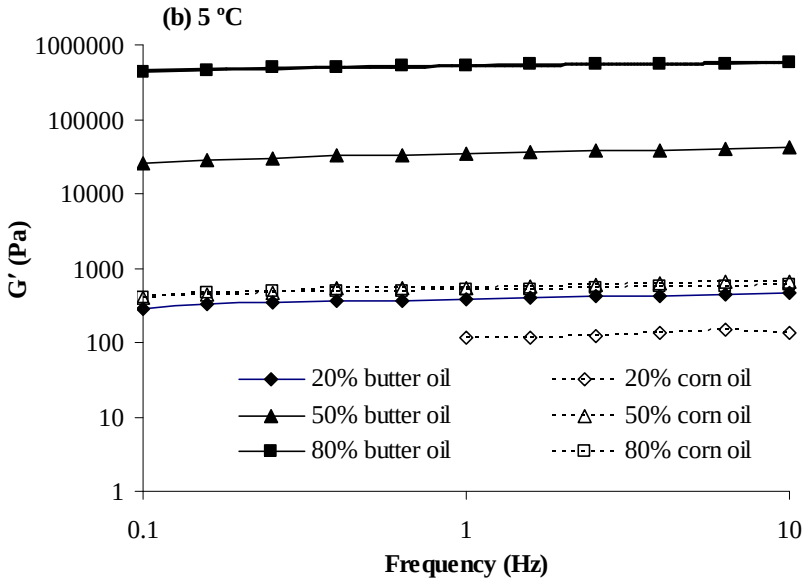
coalescence to have occurred in this emulsion during storage at lower temperature. Thivilliers, Laurichesses, Saadaoui, Leal-Calderon, and Schmitt (2008) also studied the o/w water emulsions comprising partially crystallized droplets (45% anhydrous milk fat) and stabilized by a mixture of protein and low molecular weight surfactant. The authors reported that upon cooling the droplet surface became rough and rippled as a result of the formation of irregularly shaped/oriented crystals, which could protrude into the continuous phase and subsequently coalesce upon reheating. A similar observation was also revealed in the confectionery-coating fat emulsion-xanthan mixture (Vanapalli et al., 2002). Their microscopic results indicated that upon cooling the flocs and isolated droplets crystallized and the droplet boundaries were no longer spherical. During the heating cycle, these crystalline particles started to melt, and those droplets that were in close contact with each other or otherwise began to coalesce.

As expected, the most pronounced decrease in the volume of small droplets was revealed in the 80% butter oil emulsion stored at 5 and 25 ºC, respectively. Droplet distribution data for the 80% butter oil emulsions stored at 5 and 25 ºC were not included due to a severe oil separation and samples were not stable during examining. However, the emulsion was stable and showed a good uniformity of droplet size during storage at 55 ºC and the droplet morphology was almost identical to its fresh counterparts (Figure 5.3f and 5.4b). In parallel with the emulsion stability and droplet distribution results, a fast full coalescence of emulsified butter oil droplets occurred at 25 and 5 ºC and it was able to invert the emulsion upon reheating to room temperature (Figure 5.4d and f).

*5.4.3. Viscoelastic properties*

In the present study, dynamic mechanical analysis was used to compare the rheological behavior of emulsions stabilized with tWPC. Frequency sweep profiles of freshly prepared emulsions and emulsions based on corn oil and butter oil stored at different storage temperatures were compared at various oil concentrations. In most cases, the elastic modulus ($G'$) was higher than the loss modulus ($G''$) (data for $G''$ are not shown) for all frequencies studied, indicating that emulsions behaved in a solid-gel like manner (Clark & Ross-Murphy, 1987). When the dispersed droplets were liquid, results showed that $G'$ (Figure 5.5a) and complex viscosity ($\eta^*$) (Figure 5.6a) values of corn oil emulsions were similar to those of butter oil emulsions at equal amount of oil. The dynamic mechanical moduli of emulsions showed oil concentration dependence. At higher oil fractions, the noticeably larger $G'$ and $\eta^*$ of both corn oil and butter oil emulsions were shown over an entire range of frequency. It was noticed that at higher oil content, relatively less frequency dependence was shown. The increase in $G'$ with oil concentration indicates a more solid and stronger network. An increase in the elasticity of emulsions with increasing oil content could be attributed to the droplets repulsion and deformation. With increasing oil content, droplets are packed more closely as demonstrated by the CLSM imaging. If the droplets are assumed to be rigid, non-deformable spheres, there is an upper limit of 64% ($\varphi^* = 0.64$) for random close packing of monodisperse droplets (Scott, 1960). However, our system is a polydisperse emulsion. Therefore, the figure would be greater since the smaller droplets can be packed in the void spaces formed by the packing of larger droplets. In our case, the 80% oil concentration is in this random close-packing range and hence, strong inter-droplet interactions are expected. In addition, Dimitrova and Leal-Calderon (2004) also mentioned that since the droplets are fluid, they are susceptible to deformation. It means that emulsions can be concentrated up to volume fractions


(a) Fresh
10000
1000
100
10
1
G′ (Pa)
0.1
1
10
Frequency (Hz)
20% butter oil
50% butter oil
80% butter oil
20% corn oil
50% corn oil
80% corn oil


(b) 5 ºC
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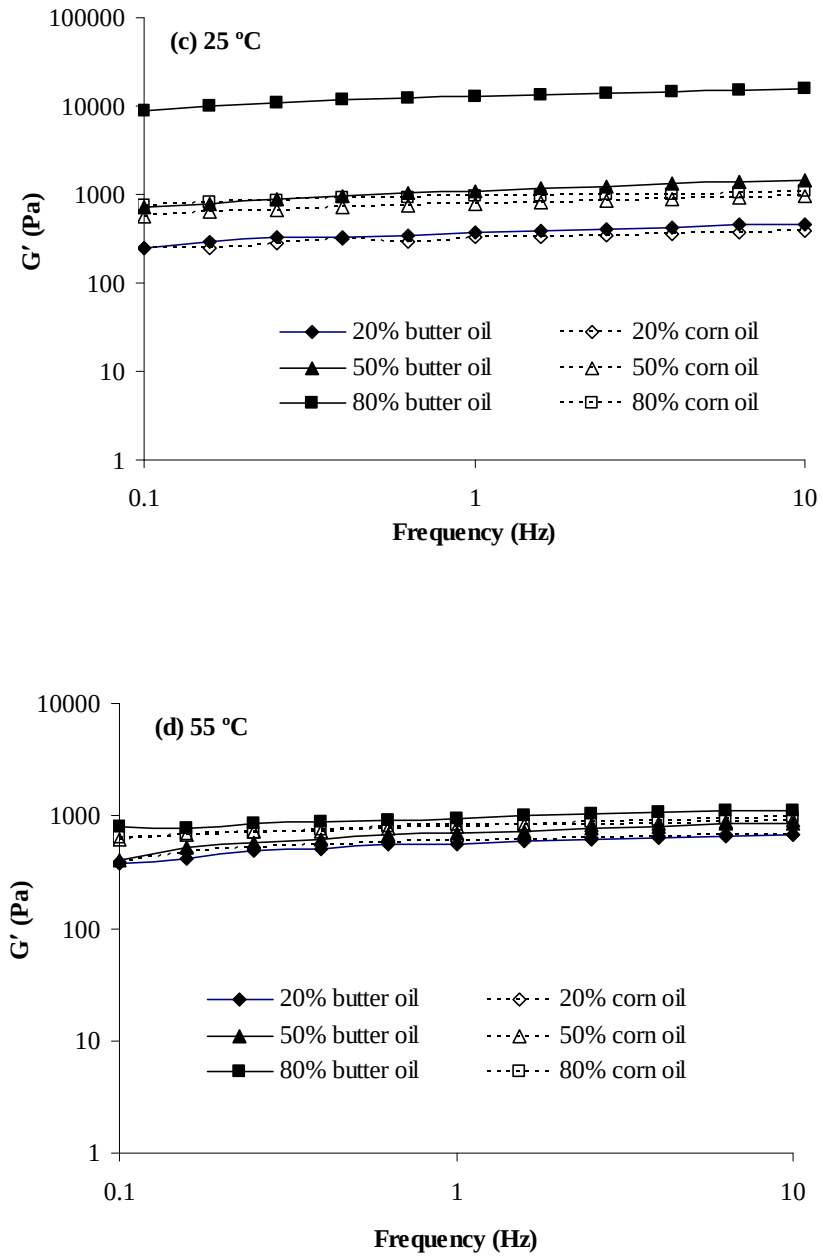
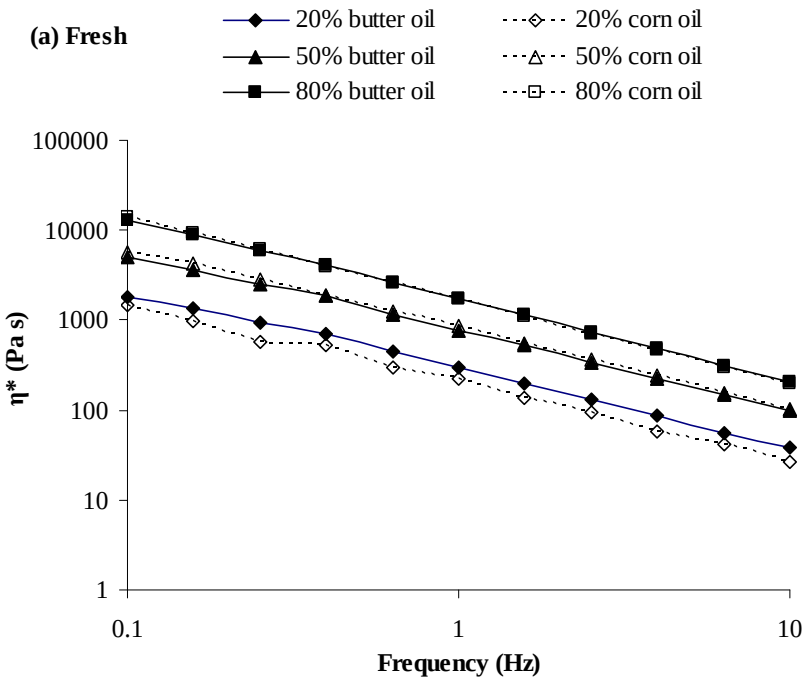
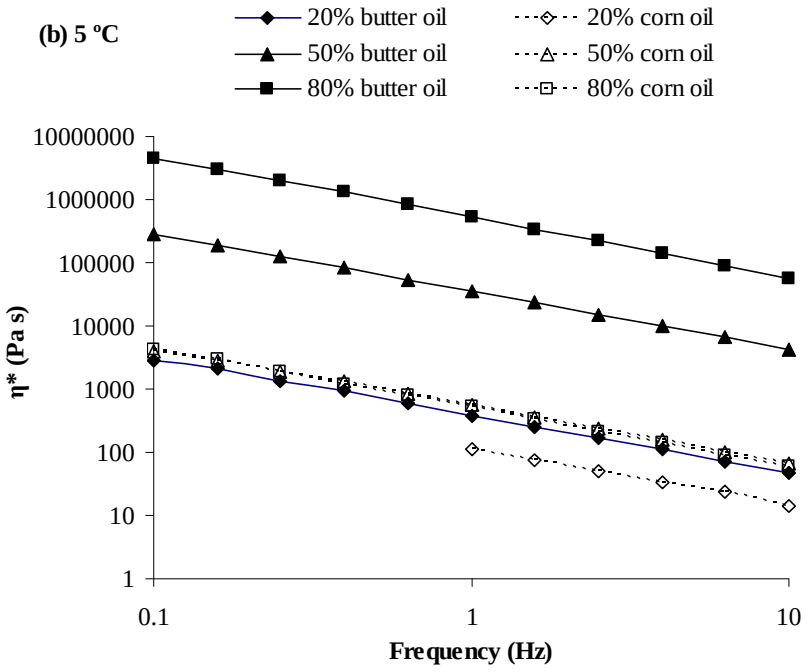



Figure 5.5. Variations of elastic modulus (*G′*) with frequency for freshly prepared (a) and stored emulsions at 5  (b), 25 (c), and 55 °C (d). Data are reported as an average of 3 replications.


(a) Fresh
20% butter oil
20% corn oil
50% butter oil
50% corn oil
80% butter oil
80% corn oil
100000
10000
1000
100
10
1
η* (Pa s)
0.1
1
10
Frequency (Hz)


(b) 5 ºC
20% butter oil
20% corn oil
50% butter oil
50% corn oil
80% butter oil
80% corn oil
10000000
1000000
100000
10000
1000
100
10
1
η* (Pa s)
0.1
1
10
Frequency (Hz)

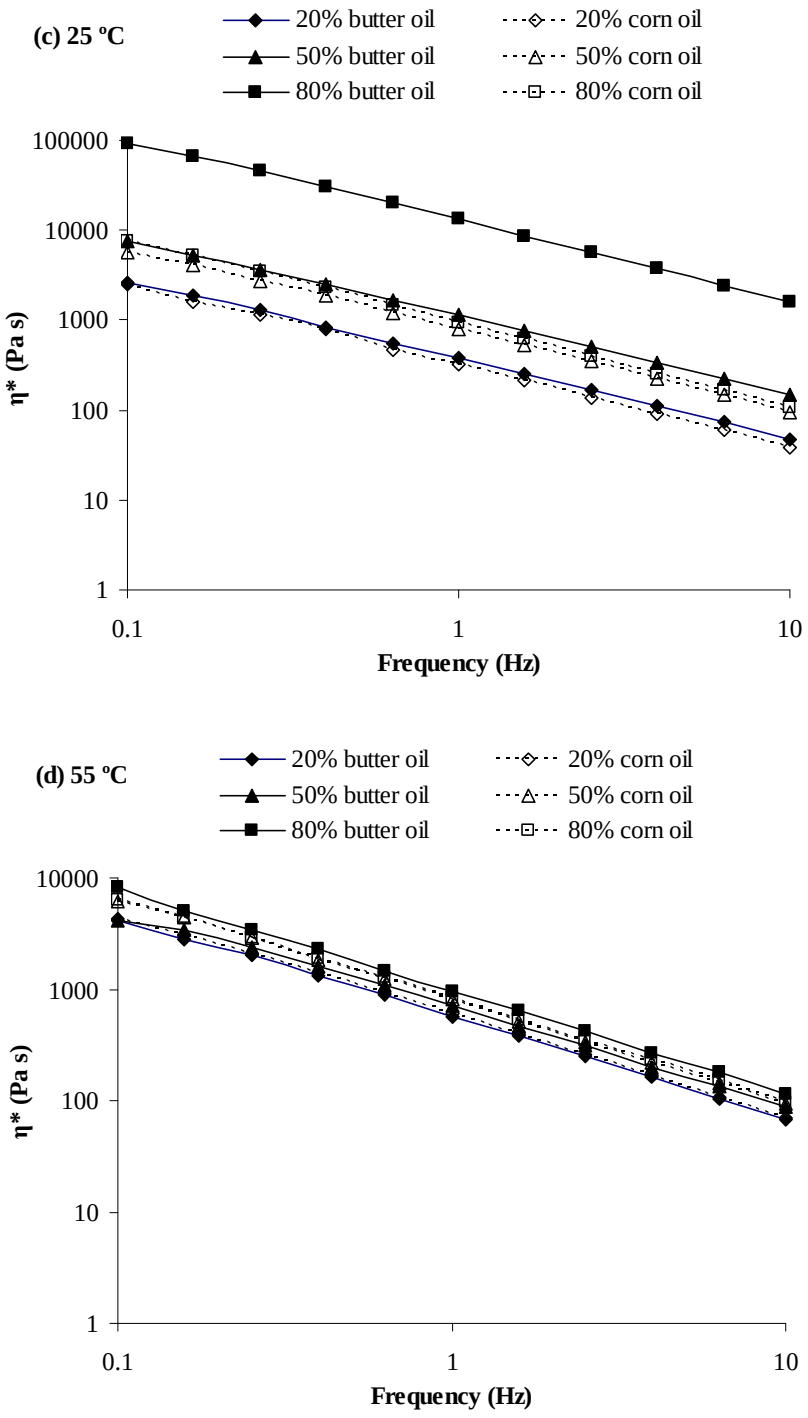



Figure 5.6. Variations of complex viscosity (η*) with frequency for freshly prepared (a) and stored emulsions at 5 (b), 25 (c), and 55 °C (d). Data are reported as an average of 3 replications.

much higher than 0.64 ($\varphi^*$). Above the volume fraction $\varphi^*$ the adjacent droplets forced together would begin to deform before their interface will actually touch due to the repulsive interactions between the droplets, and the emulsions become remarkably rigid and resemble an elastic solid. Chen and Dickinson (1998) also pointed out that the dispersed oil droplets can help to build up the gel matrix structure and significantly enhance the gel strength of protein-coated emulsions when they act as the active filler. Similar observations have been discussed in highly concentrated emulsions such as mayonnaise (Ma & Barbosa-Cánovas, 1995), hydrocolloid-stabilized emulsions (Gladwell, Grimson, Rahalkar, & Richmond, 1985; Hennock, Rahalkar, & Richmond, 1984), protein-stabilized emulsions (Dimitrova & Leal-Calderon, 2004; Hemar & Horne, 2000; Raymundo, Franco, Empis, & Sousa, 2002), heat-set WP-stabilized emulsion gels (Chen & Dickinson, 1998), and cold-set WP-stabilized emulsion gels (Boutin, Giroux, Paquin, & Britten, 2007; Rosa, Sala, Van Vliet, & Van De Velde, 2006; Sok Line, Remondetto, & Suburade, 2005).

Power law indices relating rheological variables ($G´$ and $\eta^*$) to frequency ($\omega$) over the range of temperatures studied were determined (Table 5.3) using the following relationships:

$$G' = G_0^{'}\omega^{n'} \quad (1)$$

$$\eta^* = \eta_0^*\omega^{n*} \quad (2)$$

where $G_0^{'}$ and $\eta_0^*$ represent the values of $G'$ and $\eta^*$, respectively at a frequency of 1 Hz.

Results show that corn oil emulsions were far less sensitive to variations in storage temperature than emulsions based on butter oil. All emulsions based on corn oil displayed little variation in $G´$ (Figure 5.5b and c) and $\eta^*$ (Figure 5.6b and c) during storage at 5 and 25 ºC for 7 days. However, it was noticed that $G´$ (Figure 5.5d) and $\eta^*$ (Figure 5.6d) for the 20% corn oil emulsion which contained highest

amount of protein (16% w/w) increased by approximately two times at 55 ºC. This could be attributed to the additional unfolding and denaturation of native WPs remaining in the aqueous phase during quiescent storage at 55 ºC for 7 days. Apparently, the rheological behavior of emulsions with 50 % (containing 10% protein) and 80% (containing 4% protein) corn oil was not significantly influenced during storage at 55 ºC, and it was identical to that of fresh samples (Figure 5.5a,d for $G'$ and Figure 5.6a,d for $\eta^*$). It is because the lower amount of proteins was available in the aqueous phase at increased oil concentration in the system and larger fraction of proteins was needed to saturate all the surfaces of the droplets.

Notably, temperature markedly affects the consistency of butter oil emulsions. The $G_0^{'}$ and $\eta_0^*$ values increased as temperature decreased, indicating greater elastic components at lower temperatures (Table 5.3). These results could be explained by the crystallization of fat at lower temperature, which resulted in behavior more like that of solids. The solid fat content was responsible for the rheological behaviors of emulsions. The relationship between the solid fat content and temperature (0 to 40 °C) of anhydrous milk fat was presented by Kaylegian and Lindsay (1992). Their results showed a significant increase in solid fat content when the temperature reached 5 °C (~ 55%). At 5 °C, the anhydrous milk fat contains about 50% solid fat. A much less solid fat content is revealed at 25 °C (~ 7%) and the fat crystal was completely melted at 40 °C. Table 5.3 indicates that at 25 °C both $G_0^{'}$ and $\eta_0^*$ values increased by approximately 1.3, 1.4, and 7.5 times for emulsions containing 20, 50, and 80% butter oil, respectively. The most pronounced changes in rheological behaviors of butter oil emulsions were presented at 5 °C. The $G_0^{'}$ and $\eta_0^*$ values increased by approximately 46 and 308 times for emulsions containing 50 and 80% oil, respectively (Table 5.3). Interestingly, for 20% butter oil emulsion slightly changed at 5 °C was observed, which indicates that the average distance between fat

droplets is too large for the establishment of fat crystal bridges. Clearly, the $G_0^{'}$ and $\eta_0^*$ significantly increased with an increase in oil concentration under the same storage condition. At refrigeration temperature, the 80% butter oil emulsion had a firm texture and poor spreadability, while the 50% butter oil emulsion was much softer and spreadable. It was again associated with greater fat crystallinity and solid fat content in emulsions with higher oil concentration. At 55 °C, all stored emulsions with butter oil behave similarly to those of fresh samples and stored emulsions with corn oil. It is the fact that the emulsified oil remained liquid throughout the storage period, which had little effects on droplet size and rheological characteristics of emulsions. On the other hand, the instability in rheological behaviors of butter oil emulsions during quiescent storage at lower temperatures could be one of the major reasons that caused significant droplet coalescence in emulsions, as previously presented.

Flow behavior indices, $n'$ and $n^*$, determined the frequency dependence of $G'$ and $\eta^*$, respectively. In case of corn oil emulsions, both $n'$ and $n^*$ values slightly varied with temperatures (Table 5.3). However, the $n'$ values obviously decreased for all butter oil emulsions during quiescent storage at 5 °C, showing that $G'$ became less frequency dependent and behave more like that of solid at lower temperature. The negative $n^*$ values indicate declining $\eta^*$ values with increasing frequency.

Table 5.3. Power law parameters of freshly prepared and stored emulsions based on corn oil and butter oil at different oil contents.

| Oil contents (% w/w) | Storage temperatures | Power law parameters[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Corn oil | | | | Butter oil | | | |
| | | $G'_0$ ($Pa^{n'}$) | $n'$ | $\eta^*_0$ ($Pa \cdot s^{n^*}$) | $n^*$ | $G'_0$ ($Pa^{n'}$) | $n'$ | $\eta^*_0$ ($Pa \cdot s^{n^*}$) | $n^*$ |
| 20 | Fresh | 196.71 | 0.142 | 201.48 | -0.861 | 280.37 | 0.141 | 285.03 | -0.858 |
| | 5 ºC | 112.02 | 0.108 | 113.33 | -0.893 | 378.15 | 0.094 | 384.97 | -0.908 |
| | 25 ºC | 314.98 | 0.094 | 317.77 | -0.908 | 362.03 | 0.121 | 368.85 | -0.882 |
| | 55 ºC | 571.30 | 0.106 | 581.45 | -0.903 | 544.18 | 0.119 | 557.99 | -0.896 |
| 50 | Fresh | 802.96 | 0.120 | 816.61 | -0.884 | 740.44 | 0.139 | 753.10 | -0.864 |
| | 5 ºC | 538.36 | 0.101 | 545.41 | -0.900 | 34275 | 0.097 | 35200 | -0.908 |
| | 25 ºC | 770.61 | 0.104 | 781.15 | -0.899 | 1068 | 0.149 | 1097.20 | -0.856 |
| | 55 ºC | 780.72 | 0.078 | 785.25 | -0.922 | 668.46 | 0.139 | 677.41 | -0.863 |
| 80 | Fresh | 1664.42 | 0.073 | 1675.96 | -0.929 | 1686.23 | 0.096 | 1701.76 | -0.906 |
| | 5 ºC | 507.35 | 0.066 | 510.82 | -0.935 | 519662 | 0.050 | 524848 | -0.952 |
| | 25 ºC | 933.61 | 0.067 | 939.82 | -0.935 | 12588 | 0.114 | 12851 | -0.894 |
| | 55 ºC | 803.25 | 0.089 | 813.05 | -0.913 | 948.17 | 0.085 | 961.14 | -0.919 |

[a] Data are reported as an average of three replications for each sample.

## 5.5. Conclusions

In summary, incorporation of tWPC in aqueous phase retarded droplet coalescence in all emulsions containing liquid oil upon storage. Corn oil-in water emulsions stabilized by tWPC remained stable to droplet coalescence after they were subjected to different storage conditions. The freshly prepared emulsions with monomodal droplet size distribution and narrow range of droplet size could be produced. Emulsions with higher elasticity were obtained by raising the oil concentration since oil droplets acted as active filler and strengthened the emulsions. As a result, the tWPC dispersion imparted the thick continuous phase that prevented droplet coalescence by trapping the oil droplets within the gel matrix. Another possible ability of tWPC in stabilizing emulsions could be the adsorption of the tWPC at the oil-water interface and acted as a protective coating for oil droplets. These mechanisms reduced droplet mobility and collision frequency. However, storage temperatures and oil contents markedly affected the stability and rheological behaviors of emulsions contained crystallizable oil. The crystallization of fat during storage at lower temperatures created emulsions of greater elasticity and a full coalescence appeared in emulsions containing 80% butter oil upon reheating at 40 °C. Partial coalescence was also observed in 50% butter oil stored at 5 °C, while the 20% butter oil emulsions were all stable to droplet coalescence upon storage. Thus, further study should be carried out for a deeper understanding of crystallization behaviors of emulsified fat. In addition, the mean size of the droplets, proportion of solid fat in dispersed phase, storage temperature, oil content, and the nature of droplet-droplet interactions could be the major keys in controlling the stability as well as the rheological behaviors of o/w emulsions.

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