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Formation and characterisation of soluble whey protein aggregates for their use as an emulsifier and as a rheology modifier

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Salil Wagh

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ABSTRACT

Whey protein is widely utilized in the food industry as a functional ingredient for several applications like foaming, emulsifying, gelling and encapsulating agents. Soluble whey protein aggregates can be defined as colloidal intermediates between native proteins and insoluble precipitate of gel network. A significant body of work created in the last two decades has helped us to understand the role of essential process parameters like heating temperature, duration of heating, pH, ionic strength and protein concentration in the formation and characteristics of soluble whey protein aggregates. The heightened interest in soluble whey protein aggregates is because it expands the functionality of whey proteins by allowing it to be used as a rheology modifier and stabiliser in O/W emulsions and foams. Such a value-added functionality would allow soluble whey protein aggregates to be extensively used in food applications, especially high protein and low-fat food emulsions and emulsion gels. However, the research work on use of soluble whey protein aggregates in O/W emulsions is much less explored compared to studies on the formation of soluble whey protein aggregates. The key objective of the present experimental work was to demonstrate the use of soluble whey protein aggregates in protein rich O/W emulsions and improve our understanding on the influence of soluble whey protein aggregates on the characteristics of O/W emulsions in terms of physical properties, storage stability and stability against thermal treatment. A range of process conditions like heating temperature (68, 75 and 85°C), duration of heating (up to 60 min) and pH (6.9, 6.4, and 6.0) were investigated to understand their impact on the characteristics and yield of soluble whey protein aggregates. Soluble whey protein aggregates with varying particle size (40 nm to 130 nm) were developed and they were further utilized to prepare O/W emulsions with constant oil content (15% w/w) and varying oil content (10 to 30% w/w) to understand its impact on the physical characteristics and stability of O/W emulsions. This experimental study is an endeavour to build a toolkit to prepare high protein low fat emulsions and emulsion gels based on soluble whey protein aggregates.

Keywords: *soluble whey protein aggregates, emulsions, rheology modifier, high protein*

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Chapter 1. Introduction

Increasingly, modern consumers do not look at food merely from a utilitarian point of view of satiating hunger. Food is also expected to serve as an effective tool to improve health. While protein has always been an important macronutrient, its importance in public discourse has been on the rise in the recent decades. The notion has not only been propagated by modern marketing machinery but also has been unequivocally proven as a superior nutrient by several clinical studies on assimilation within human body. The modern consumer is spoilt for choice with marketplace cramped with proteins from different sources – plant, muscle and dairy. The choices have emerged as modern food companies and food formulators try to address an elevated demand for high protein food products. There is an equally strong demand for reduced fat products. Although much smaller in its quantum, there is a burgeoning demand for "clean label" food products. There is a sumptuous body of research work which has been undertaken in the recent decades to investigate several food proteins to effectively address each of these demands.

Whey proteins are one of the most widely used sources of protein and a functional ingredient in the food industry. For a food technologist, whey proteins are a vital functional ingredient due to its use as an emulsifying agent, a foaming agent and a gelling agent. There has been an endeavour of several food technologists and colloidal scientists to modify whey proteins to enable its use as a rheology modifier or a fat replacer without sacrificing its existing properties. The present experimental work was undertaken to enable use of whey proteins in high protein and reduced fat emulsions. Whey protein aggregates soluble in water (henceforth referred to as ***soluble whey protein aggregates***) are a colloidal form of whey proteins achieved by thermal treatment of whey protein solution which has been gaining interest in recent times. The present study is not only successful in establishing process parameters needed to achieve a high yield of aggregates but also demonstrates that such whey protein particles can be effectively used as rheology modifiers. The present thesis is divided into six chapters, including this Introduction chapter.

Chapter 2 is a review of literature published in the recent and distant past to understand and acknowledge the importance of whey proteins as a nutrient and as a functional ingredient. It briefly describes its origin, manufacturing process and key properties which make it one of the most popular food proteins in the world. The chapter reviews studies conducted in the past on preparation of soluble whey protein aggregates from the point of view of process kinetics and influential process parameters. A large flotilla of food products which occupy shelves in supermarket, mom-and-pop stores, refrigerators in our homes and food service chains can be categorized as emulsions or emulsion gels. These would include milk, coffee creamer, cream, yogurts, cream cheese, salad dressings, whipped toppings, mayonnaise, sauces, and ice creams. Soluble whey protein aggregates have been used in recent years to harness its use as an emulsifier and a rheology modifier. Its ability to influence rheology also has substantial importance in the products with a "clean label". However, merely formation of O/W emulsions would not be sufficient to translate its use to real world applications. A detailed review of environmental and process conditions on the stability of such emulsions is covered in this chapter.

Chapter 3 presents the research work undertaken for the formation of soluble whey protein aggregates. A solution of soluble whey protein aggregates (8% w/w) was prepared by varying the values of three process parameters, such as heating temperature, duration of heating and pH of the solution. The significant effects were noted by measuring key physical characteristics including particle size, ζ-potential and solubility (yield). Based on the results, a set of process parameters was chosen to prepare soluble whey protein aggregates with varying particle size. These particles were investigated further for their use as an emulsifier and a rheology modifier in Chapters 4 and 5.

Chapter 4 presents the experimental work undertaken to understand the effect of particle size of soluble whey protein aggregates on the characteristics and stability of O/W emulsions. The concentrations of protein and oil used were chosen in cognizance with the present regulation and guidelines laid down for high protein and reduced fat versions of popular O/W emulsions. Soluble whey protein aggregates of varying particle size (40 to 150 nm) were obtained by process parameters selected from the results of the study discussed in Chapter 3. The characteristics of O/W emulsions such as droplet size, ζ-potential and apparent viscosity were measured to determine the effect of particle size of whey protein aggregates. Stability of O/W emulsions was also determined based on the measurement of droplet size on storage, change in pH, change in ionic strength and thermal treatment of emulsions. Based on the results, soluble whey protein aggregates obtained by heating at near neutral pH were selected to investigate further its role as an emulsifier and as a rheology modifier.

Chapter 5 presents the laboratory work conducted to investigate the effect of oil content on the characteristics and stability of O/W emulsions stabilised by soluble whey protein aggregates. Soluble whey protein aggregates with an average particle size of approx. 50 nm was obtained by heating a solution of whey protein isolate at near neutral pH. The characteristics of resulting O/W emulsions such as droplet size, zeta (ζ)-potential and apparent viscosity were measured to determine the effect of oil content. Stability of O/W emulsions was determined by measurement of droplet size during storage and thermal treatment of emulsions was also investigated for its effect on emulsion stability.

Chapter 6 provides a comprehensive analysis of conclusions drawn from the experimental work on the formation of soluble whey protein aggregates and its subsequent use as an emulsifier and a rheology modifier. The research work would provide an important toolbox to construct high protein and reduced fat O/W emulsions based on soluble whey protein aggregates.

Chapter 2. Literature Review

2.1 Introduction

Whey protein occupies an important place in the pantheon of food ingredients. It is known for its diverse roles in food formulation and daily consumption. These include its use as a functional (emulsifier, foaming agent, stabiliser) (Dickinson, 1994; Hoffmann & Reger, 2014; McClements, 2004), nutritional (energy source, physiological metabolism) (Beelen et al., 2017; Norton et al., 2021), and medicinal ingredient (anti-viral, wound healing, anti-inflammatory) (Chatterton et al., 2013; Smithers, 2008; Zhou et al., 2015). A number of review articles and studies have demonstrated the use of whey proteins in baby food (Fenelon et al., 2019; Gan et al., 2018), nutrition bars/solid meals (Hertzler et al., 2020; Lu & Zhou, 2019; Małecki et al., 2020), functional foods (Fritsch et al., 2017; Kelly, 2019; Sharma, 2019; Sim et al., 2020) and sports supplements (Ali et al., 2019).

Whey proteins are extensively used in several food structures as a functional ingredient owing to their ability to adsorb at the interface as an emulsifier or foaming agent (both air and oil can be treated as hydrophobic) and for their gelling and water-holding. While the former allows for formation and stabilisation of useful food structures – emulsions, emulsion gels, foams, batters, the latter has important ramifications on textural and sensory properties of the food products (Singh & Ye, 2020). The structural and compositional aspects of whey proteins contribute immensely to its functionality. Its amphiphilic nature allows it to not only adsorb at the interface but also keeps it sufficiently solvated by the surrounding aqueous phase to stabilise the emulsion/foams. For a thermodynamically unstable system like O/W emulsions, the interfacial layer provides the necessary kinetic stability to the emulsion. Any modification to the emulsion which brings about undesirable changes at the interfacial layer would destabilise the emulsion (Dickinson, 1992a, 1998). O/W emulsions often destabilise due to one of the following mechanisms - coalescence, flocculation, creaming, gravitational sedimentation and Ostwald ripening (D.J. McClements, 2015).

In recent years, there has been a steady rise in a consumer preference towards clean label (Asioli et al., 2017; Ozturk & McClements, 2016). This has prompted several food manufacturers and food technologists to look for ingredients which do not bear an E number. A hunt for natural ingredients such as alternative protein-based emulsifiers, stabilisers, foaming agents and thickeners has gained significant interest among food scientists (Famelart et al., 2015; McClements et al., 2017; Setiowati et al., 2020) as these accompany a GRAS status (de Castro et al., 2017). For example, protein particles based on whey proteins, which could not only improve emulsion stability against coalescence, thermal treatment, and change in pH and ionic strength but also provide desirable emulsion rheology, have remained a subject of significant interest since a few decades ago. **Soluble whey protein aggregates** or colloidal whey protein aggregates soluble in water is one such whey protein particle which has shot to prominence with a range of studies on formation and quantification of useful properties like surface hydrophobicity, emulsifying activity index, foaming stability and rheology (Knudsen et al., 2008; Moro et al., 2001; Schmitt et al., 2007; Vardhanabhuti & Foegeding, 1999). There have also been a few studies in the past to understand their use in O/W emulsions and emulsion gels and their impact on its characteristics (Boutin et al., 2007; Chevallier et al., 2016; Dybowska, 2011)

This literature review covers - nutritional, sensory and functional properties of whey proteins to understand the importance of whey protein as a food ingredient. The present chapter conducts a detailed review of several studies in the past on to understand kinetics of denaturation and aggregation of whey proteins in formation of soluble whey protein aggregates. There are several important process parameters like protein concentration, pH, ionic strength, heating temperature and duration of heating which have shown to influence the characteristic of soluble aggregates. The parameters have also been reviewed in detail. The review tries to set the stage for the project undertaken to build an oil-in-water platform (emulsion and emulsion gels) based on whey protein aggregates. Such a platform would be an effective tool to prepare high protein-low fat food structures for a wider consumer appeal.

2.2 Whey protein

2.2.1 Nutritional aspect of whey protein

Proteins are complex macromolecules or biopolymers composed of “amino acids” The composition and structural arrangement of amino acids determine the functional and nutritional properties of proteins. Whey protein has been found to be considerably better than other protein supplements like casein or soy protein in its ability to prevent muscle damage in critically ill patients (Marik, 2015), elderly (Burd et al., 2012; Pennings et al., 2011), and younger population (Macnaughton et al., 2016; Tang et al., 2009). As an example, the content of leucine, which is an essential amino acid for muscle building and anti-inflammation after workout, is almost two times more than that of soy protein known as the second highest in leucine content among a number of proteins from different sources. Leucine content in whey protein isolate at 14 g/100 g of protein is much higher compared to comparable quantity of other protein sources – 18 g in muscle protein and 8 g in soy protein (Ali et al., 2019; Layman, 2003). Food and Agriculture Organisation (FAO) has suggested evaluation of protein on the basis of three parameters, such as amino acid composition, digestibility and biological scores. Essential amino acid (EAA) score and digestible indispensable amino acid score (DIAAS) have been used to determine and compare the nutritional quality of proteins from different sources (FAO, 2011; Hoffman & Falvo, 2004). Whey protein is known to have a much higher DIAAS score than other milk (e.g. casein)/animal proteins (e.g. beef protein) and plant-based proteins from different sources like wheat flour, pea protein, soy protein, etc. The only protein to have scored slightly better than whey protein is egg protein (Sarwar, 1997).

Several prominent food proteins, such as meat, egg albumin, whey protein, casein, soy pea protein and wheat gluten, have been evaluated for their satiating effect. Some of the prominent parameters used for evaluation are plasma amino acid levels (including postprandial in some studies) and insulin response or insulinotropic effect. Whey protein has been found to better than meat proteins, (e.g., tuna, fish, beef), egg albumin (Lang et al., 1998; Pal & Ellis, 2010) and casein (Pal et al., 2014; Pennings et al., 2011). Whey protein has also demonstrated much faster digestion and absorption rates compared to other form of milk protein – casein (Boirie et al., 1997; Dangin et al., 2001) Whey protein is now considered as a “fast” protein. Popular food systems containing whey protein, range from low viscosity emulsions (milk, beverages, chocolate milk) to viscous (salad dressings, soups, cream cheese) to viscoelastic gels (desserts, yogurts) Recent studies have shown that structure of products determine the ability of proteolytic enzymes (pepsin) to access proteins and speed of hydrolysis (Luo et al., 2021; Luo et al., 2015). Food structures with intricate networks (gels) have shown slower rate of gastric digestion than fluids (Guo et al., 2014).

There have been several attempts in recent past to assess consumer acceptance of protein fortified jams, breads, cakes, biscuits, yoghurts etc (Ahmed et al., 2019; Ammar et al., 2021; Karam et al., 2013; Mulders, 2017). In case of popular products like yogurts, the presence of whey protein and its proportion with other milk protein, casein strongly affects gelation pH, hardness of gel which would strongly influenced creaminess and chewiness of yogurt (Vasbinder et al., 2004). These studies have often noted a negative perception of graininess or chalkiness in the products with higher quantities of whey proteins. While some customers are willing to overlook these negative attributes, it remains a challenge to include higher amount of whey protein in popular food products.

2.2.2 Chemical composition, structure, and properties of whey protein

Milk proteins can be broadly divided into caseins and whey proteins. Whey proteins are defined as a fraction of milk proteins which remains soluble in serum phase after the precipitation of caseins when the pH of milk is adjusted to pH 4.6. The pH 4.6 is thus known as the isoelectric point of caseins. The content of whey proteins in bovine milk represent about 20% of the total milk protein. Whey proteins can be further divided into major and minor whey proteins. Major whey proteins include β-lactoglobulin (β-lg), α−lactalbumin (α-la), bovine serum albumin (BSA), and immunoglobulins (Ig). Minor whey proteins include lactoferrin (LF) and glycomacropeptide (GMP) represent a mass fraction of less than 0.5% of whey proteins and do not contribute materially to the functional properties of commercial whey proteins (Deeth & Bansal, 2019). The proportion of major whey proteins depends on the source and method of manufacturing of whey proteins. **Table 2.1** shows the distribution of major whey proteins in some whey proteins products. This indicates that β–lg is the dominant component of most whey protein powders.

Whey proteins are structurally globular proteins with hydrophobic amino acids mainly buried in the interior of their molecular structure, making whey proteins soluble in water as their surface area is mainly covered by hydrophilic amino acids. This implies that hydrophobic interaction is one of the main factors that enable whey proteins to form the globular conformational structure (Bigelow, 1967; Schmidt et al., 1984)

Table 2.1 Mass distribution* of major whey proteins in commercial whey protein products

PRODUCT	β-lg	α-la	BSA	Ig
Sweet whey	3.23	0.79	0.12	0.48
Demineralised (90%) whey	32.06	18.94	1.45	0.23
Cheese WPC	30.65	10.91	1.13	3.34
MF-WPI	40.55	12.55	1.17	3.54
IE – WPI	70.66	10.8	2.59	3.25

* Expressed as % dry basis
WPC: whey protein concentrate, WPI: whey protein isolate, MF: Micro-filtration, and IE: Ion-exchange
Source: (Amundson et al., 1982; Boland, 2011; Elgar et al., 2000)

Some physicochemical properties of major whey proteins and lactoferrin are shown in **Table 2.2,** including their isoelectric point (pI) and denaturation temperature (T_m). The structure of β–lg is characterised by the presence of two disulfide bonds and one free-SH group along with nine anti-parallel β-sheets and C-terminal α-helix (Papiz et al., 1986; Surroca et al., 2002). The structure of β–lg has been extensively studied under several pH conditions using X-ray crystallography and Nuclear Magnetic Resonance (NMR) spectroscopy (Fogolari et al., 1998; Papiz et al., 1986; Qin et al., 1998). In case of α-la, it is characterised by the presence of calcium linkage (holo form) that leads to the substantial difference denaturation temperature of α-la compared to the apo form (without calcium) (Edwards & Jameson, 2020). T_m for holo form is higher 65°C compared to 35°C for apo form (Boye & Alli, 2000). Inclusion of calcium linkage however does not lead to a significant difference in the structure of apo and holo forms.

The structural changes induced by changes in pH and temperature have been used to form suitable whey protein aggregates with improved functionality for their applications in O/W emulsions, emulsion gels and foams. Whey proteins are an important functional ingredient due to their water solubility and emulsifying, gelling and foaming properties. They are therefore widely used in food formulations and structures owing to their ability to adsorb at the interface as an emulsifier or foaming agent and also to form gels with water-holding capacity. These functional properties enable whey proteins to stabilise and modulate the textural and sensory properties of many food systems.

Table 2.2 Important physicochemical properties of major whey proteins and a minor whey protein (lactoferrin)

Whey protein	% Mass (dry basis)	Molecular weight (kDa)	pI	T_m*	Number of disulfide bond/free SH group	Absorptivity at 280 nm**
β−lactoglobulin	50-60	18.4	5.2	72	2/1	9.6-10
α−lactalbumin	15-20	14.2	4.4	35 & 64	3/0	20.1-20.9
Bovine serum albumin	3	66.3	4.9	64	17/1	6.3-6.9
Immunoglobulin	10	90-1000	-	-	-	12.1-13.6
Lactoferrin	<0.1	80	8-9	70 & 90	17/0	9.91

* Denaturation temperature at neutral pH, ** Calculated at 1-cm path length

Source: (Deeth & Bansal, 2019; Edwards & Jameson, 2020; Farrell et al., 2004; McClements & Grossmann, 2021)

2.3 Whey protein aggregates

Whey protein particles labelled as "soluble whey protein aggregates", "whey protein polymers", "whey protein nanoparticles", "whey protein microgels", "microparticulate whey protein (MWP)" or "partially denatured whey protein products (PDWNP)" have been claimed as suitable candidates for use as a "fat replacer" (Kew et al., 2020; Liu et al., 2016). A use of whey protein particles as a "fat replacer" provides a dual benefit. It not only helps to reduce fat (and calories) but also improves the protein content of the product. Most of the strategies to form whey protein-based fat replacers rely on thermal treatment of whey protein solutions/dispersions (Ako et al., 2009; Nicolai & Durand, 2013; Ryan et al., 2013; Schmitt et al., 2010). Thermal treatment of whey protein in solution leads to protein denaturation and aggregation due to unfolding of its initial folded structure. **Table 2.3** shows a list of patents related to whey protein particles produced under different environmental conditions for their applications. The theoretical models and recent studies on the formation of whey protein aggregates are described in further detail in subsequent sections of this literature review.

While denaturation of whey protein involved in forming whey protein aggregates leads to loss of its biologically active or native state, it can induce a viable functional ingredient in food formulations (van Lieshout et al., 2020). In fact, thermal treatment of whey protein improves its physiological importance. As noted by some studies on WPC powders (Lindberg et al., 1998), WPI dispersions (Wang & Zhao, 2017), WPI gels (Lorieau et al., 2018; Singh et al., 2014), and β-lg dispersions (Dupont, Boutrou, et al., 2010; Guo et al., 1995; Loveday et al., 2014), denatured whey proteins, especially β-lactoglobulin, show extensive and rapid hydrolysis in gastric and intestinal phases. Some recent studies on infant gastro-intestinal tract have also concurred with these conclusions (Dupont, Mandalari, et al., 2010).

2.3.1 Soluble whey protein aggregates

Thermal treatment of whey protein above its denaturation temperature leads to formation of “soluble” as well as “insoluble” aggregates due to denaturation and aggregation of protein (Dannenberg & Kessler, 1988; deWit & Klarenbeek, 1984; Kinsella & Whitehead, 1989). Solubility of whey protein is a physical property which would indicate an important structural state of whey protein as depicted in **Figure 2.1**. In recent years, the studies on whey aggregates have resorted to measurement of solubility at pH 4.6 to ascertain the degree of denaturation (De Wit, 1990; Kehoe et al., 2011; Ryan et al., 2012; Zúñiga et al., 2010). Solubility also indicates colloidal state of aggregates formed after denaturation of whey proteins (native, partially denatured or completely denatured) after thermal treatment (Loveday, 2016).

Solubility has been used to ascertain colloidal stability of whey protein aggregates formed on thermally induced denaturation by (Britten & Giroux, 2001; Gulzar et al., 2012; Hudson et al., 2000; Mehalebi et al., 2008). In such a scenario, as depicted in **Figure 2.1,** solubility would be a measure of amount of soluble whey protein aggregates as well residual native whey protein. While reviewing the literature on the subject, a distinction has been noted where solubility has been used as a measure of denaturation. While reviewing such literature or studies, emphasis has been laid on the data available for size and molecular mass of aggregates measured by SDS-PAGE, size exclusion chromatography – multiple angle laser light scattering (SEC-MALLS) and Reversed phase-HPLC to infer the colloidal

Table 2.3 List of some selected patents on preparation and use of whey protein particles

PRODUCT NAME	REFERENCE	DATE	CURRENT ASSIGNEE/ INVENTORS	PROMINENT FEATURE
Protein Product	EP0323529B1 USP 4,734,287	02/12/1992, 29/03/1988	John Labatt Limited	Macro-colloid with size of 0.1 µm to 2 µm. 90% of soluble protein particle.
Whey protein vehicle for active agent delivery	USP 9,198,445	01/12/2015	Nestec S.A.	Protein micelle size 100 – 900 nm. Formation at pH = 5.8 – 6 at 80°C.
Cosmetic use of whey protein micelles	USP 9,387,158	07/12/2016	Nestec S.A.	Whey protein micelle with size 100 – 900 by adjusting pH = 3.5- 9, temp – 70 to 95°C. between 12 to 25 min. Use in cosmetics.
Insoluble Protein Particles	EP1292196B1	01/08/2007	PROCTER & GAMBLE	Non-aggregated denatured protein with size 0.1 µm to 5.0 µm with 90% insoluble protein.
Aggregate whey protein product	USP 6,767,575	27/06/2004	Huss; Manfred (Freising, DE), Spiegel; Thomas (Munchen, DE)	Maximum protein content – 4%, with size of 1 to 4 µm formed between 75 to 150°C.
Process for the preparation of whey protein aggregate particles and use thereof	USP 10,548,337	04/02/2020	Nestec S.A.	Whey protein denatured and gelled at 76°C and pH = 7.1. Gelled whey protein dried and powdered to make whey protein aggregate particles. Edible foam using 1.5%.
Partially denatured whey protein product	USP 5,494,696	27/02/1996	Danmark Protein A/S	Partially denatured whey protein with protein content with 65-95% with particle size of 30 to 60 µm.
Dry Microparticulated Protein Product	WO1993007761A1	29/04/1993	NutraSweet Company	Spray-dried non-aggregated spheroidal particles of size 0.1 to 3 µm.
Cream cheese made from whey protein polymers	USP 7,250,183	31/07/2007	Kraft Foods Holdings, Inc	WPC with protein concentration of 5-20% with pH 7-9 and heating 70-95°C for undisclosed duration.

characteristics of aggregates. It may be regarded that "soluble" whey protein aggregates are a precursor of whey protein gels depending on the initial concentration of whey protein in solution. In fact, one of the early mentions of "soluble" whey protein aggregates can be found in a research work by McSwiney et al. (1994) on heat induced gels which noted that protein aggregates are pre-gel particles formed in the dispersion before the formation of an extensive gel network. However, in the context of the present experimental work, soluble whey protein aggregates refer to aggregates which form a stable colloidal dispersion in water without precipitation.

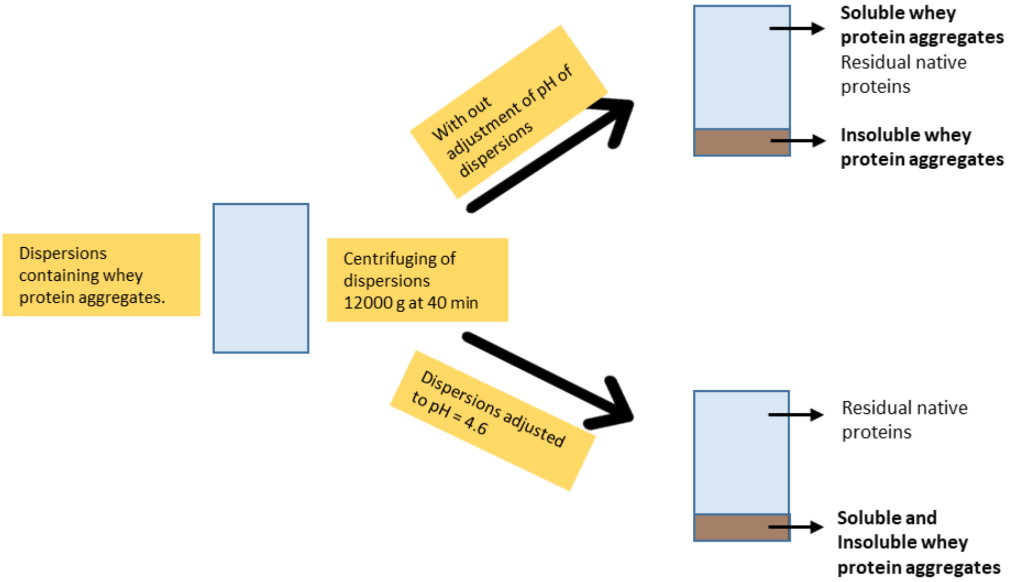


Figure 2.1 Soluble whey protein aggregates formed by heat induced denaturation and aggregation. Adapted from Gulzar et al. (2012)

2.3.2. Kinetics of thermal denaturation and aggregation

Protein denaturation can be defined as a "loss of native structure (secondary and/or tertiary) of protein without change in the molecular weight (Tanford, 1997). At a non-heated and neutral pH, whey proteins maintain a dimer-monomer equilibrium in an aqueous solution (Cairoli et al., 1994; Casal et al., 1988). For a globular protein like whey proteins, denaturation primarily involves unfolding of protein molecules which exposes free thiol and a hydrophobic patch/region (Roefs & De Kruif, 1994). This is termed as formation of a non-native intermediate (Qi et al., 1995; Qi et al., 1997). Depending upon the temperature and duration of heating, non-native intermediates could renature into native proteins. Thus, denaturation could be both reversible and irreversible. Denaturation of whey proteins can be achieved by a number of methods – thermal treatment, change in pH, high pressure, ultrasonication and gamma irradiation. In the context of the present work, denaturation induced by thermal treatment alone has been focused. Whey proteins in their native state remain stable in aqueous solution.

Formation of soluble whey protein aggregates by thermal treatment can be considered as a set of two reactions, such as denaturation and aggregation. The rate of each reaction is influenced by pH of dispersion, ionic strength, heating temperature, duration of heating and protein concentration. Subsequently several recent studies of wet heating of whey proteins as shown in **Table 2.4** have noted that structural properties, composition, physical and chemical properties of soluble whey protein aggregates are influenced by process parameters such as protein concentration, temperature, duration of heating, pH and ionic strength. The dominant portion of whey proteins, that is β-lg, is heat labile (Dannenberg & Kessler, 1988) while α−la is relatively much more stable to aggregation on denaturation due to its calcium linkage (holo form) (Wijayanti et al., 2019). A possible reason for such resistance to aggregation could be reversible denaturation or renaturation of α−la (Relkin et al., 1993). The holo form (containing calcium) has shown 100% renaturation even at high heating temperature of 90°C. Apo form which does not contain calcium has shown slightly lower values of renaturation at 61% at 90°C (Boye et al., 1997). The presence or absence also dictates the temperature of onset of irreversible denaturation of α−la. The absence of calcium lowers the onset temperature of irreversible denaturation (Hong & Creamer, 2002). It has been observed that both forms of α−la undergo > 70% irreversible denaturation for heating temperatures of > 95°C (Wijayanti et al., 2019). Such a resistance to aggregation on thermal denaturation has also been attributed to lack of free thiol radicals. Reversibility of denaturation could occur in absence of calcium (apo form) and splitting free disulphide bonds in presence of β-lg (Boye & Alli, 2000). It is safe to assume that thermal treatment of whey proteins at a temperature above denaturation temperature of β-lg (> 70°C) for a sufficient duration of time is primarily influenced by denaturation and aggregation β-lg.

Denaturation and aggregation of whey proteins has been suggested to follow a model analogous to free radical polymerisation which is comprised of three steps, such as initiation, propagation and termination by Roefs and De Kruif (1994). β-lg is present in solution as dimers at ambient conditions. Initiation step involves a series of sub-steps which involve denaturation of dimers as well as transition of dimers to monomers. Denatured dimers react with non-denatured monomers and to form reactive monomers (deWit & Klarenbeek, 1984). Reactive monomers have a reactive free thiol group which behaves like a free radical and marks the conclusion of initiation step. Propagation step leads to further formation of reactive species and formation of protein aggregates due to covalent bonds (disulphide bonds). Exhaustion of reactive monomers marks the termination step. The relevance of the model is confined to near neutral pH and for temperatures between 60 and 70°C. It implies that aggregation is stopped once free thiol radicals are unavailable. The model assumes that aggregates are formed only through covalent bonds. The model has been refined by taking into consideration the impact of process parameters like temperature and heating time on the reaction rates of denaturation and aggregation. Several environmental factors like pH, ionic strength and protein concentration have a decisive impact on reaction kinetics (de la Fuente et al., 2002; Tolkach & Kulozik, 2005; Tolkach & Kulozik, 2007; Verheul et al., 1998; Zúñiga et al., 2010). The three-step model has been altered to accommodate dimer-monomer transition before unfolding of monomers which exposes reactive thiol group and formation of "molten globule" state of whey proteins (Iametti et al., 1996; Qi et al., 1995). The model has been further refined to accommodate the role of hydrophobic or non-covalent interactions observed at heating temperatures of > 80°C (Croguennec et al., 2003; Hoffmann & van Mil, 1999; Hoffmann & van Mil, 1997; Mehalebi et al., 2008; Schokker et al., 1999). An updated schematic of the model is provided in **Figure 2.2**

Figure 2.2 A schematic representation of thermal denaturation and aggregation of whey proteins (adapted from (Hoffmann & van Mil, 1997; Roefs & De Kruif, 1994; Ryan et al., 2013)

2.3.2.1 Effect of temperature

Whey proteins comprise of several components with denaturation temperature ranging from 62°C for α-la and BSA to 75°C for β-lg. In the context of the present work, we have only considered heating temperature in the range of 65°C to 95°C. It was reported that the polymerisation reaction follows an overall order of reaction (n) = 1.5 for depletion of whey proteins (β-lg) (Dannenberg & Kessler, 1988; Roefs & De Kruif, 1994) and n= 1 for α-la (Lyster, 1970). The reaction order has been found to be in the range of 1 to 2 at different temperatures ranging from 52 to 90°C for pH 6.0 to 7 (Croguennec et al., 2004; Hoffmann & van Mil, 1999; Zúñiga et al., 2010). Depletion of native protein (C) and formation of higher order aggregates can be expressed by a first order differential equation (Equation 1).

$$\frac{dC}{dt} = k_n * C^{n} \qquad \text{(Equation 1)}$$

The reaction constant (k_n) can be expressed by Arrhenius equation as,

$$\ln k_n = \ln k_0 - {}^{E}/_{R * T} \qquad \text{(Equation 2)}$$

where E = activation energy, R = universal gas constant and T = temperature

As expressed in Equation 2, the reaction rate constant depends on temperature (T). The amount of aggregation of whey proteins can be controlled by heating temperature as well as duration of heating. For heating temperatures of < 70°C, it has been found by several experiments that a significant portion of β-lg remains in native state and limits the size of aggregates even after extensive heating time (Roefs & De Kruif, 1994). Also, it has been noted that denaturation rate increases with temperature on heating and the aggregates formed at this temperature mostly consist of covalent bonds and does not involve non-covalent interactions.

For heating temperatures of > 75°C, denaturation is considered to be irreversible. For heating temperatures of < 70°C, Tolkach and Kulozik (2007) noted that a significant fraction of unfolded proteins (α) re-folds into native state which is based on subsequent cooling of the heated protein solution. Hence, the denaturation rate should be considered a rate limiting step. Based on this study, the reaction rate constants for denaturation (k_d) and aggregation (k_{ag}) can be expressed as,

Table 2.4 Recent studies on thermal denaturation and aggregation of whey proteins by wet heating

PROTEIN	VARIABLE	METHOD	REFERENCE
β – lg	Heating temp = 61.5, 65 and 68.5 °C, pH = 6.75 to 6.95 Protein concentration = 1.39% to 9.2% Extensive heating time	• For all heating temperature overall reaction order is 1.5 • Rate of denaturation increased concentration and heating temperature. • Size of aggregate is directly proportional to the monomer concentration in the dispersion. • Denaturation and aggregation are a three-step polymerisation reaction.	(Roefs & De Kruif, 1994)
β – lg	Heating temp = 65- 80°C, Concentration = 0.9 % w/v pH = 6.0 to 8 NaCl = 0 to 100 mM. Protein concentration varied from 0.2% to 4.6% in presence of NaCl. Duration of heating = 120 min	• Kinetics of denaturation varied with temperature and pH. • Particle size of aggregate was found to be directly proportional to protein concentration and mass of aggregates. • Denaturation rate reduced with pH for heating temperature of 65, 68.5 and 75°C.	(Verheul et al., 1998)
β – lg	Heating temperature = 85 and 90 °C, pH = 2, 5.8, 7 Duration of heating = 15 min and protein concentration of 1% w/v at pH = 5.8 and 7. 300 min and 4% w/v for pH = 2	• Conversion of native proteins to aggregates >80% for all conditions • Fibrillar structure at pH =2, spherical at pH =5.8 and curved strands at pH =7 • Size of aggregates = 15 nm for pH =7 and 2. 120 nm for pH = 5.8	(Jung et al., 2008)
β – lg	Heating temperature = 80 °C, pH = 6,6.4 and 6.8 Duration of heating = 15 min Protein concentration = 5% w/v	• Rate of denaturation increases with reduction in pH. • Size of aggregates increases with reduction in pH. 40 nm (pH =6.8), 50 nm (pH = 6.4) and 100 nm (pH = 6.0). • Presence of non-covalent interactions leads to larger aggregates. • Higher insoluble aggregates with reduction in pH.	(Zúñiga et al., 2010)
WPI	Heating temperature = 85 °C, pH = 6.0 to 7.0 NaCl = 0 to 120 mM	• Rate of denaturation increases with reduction in pH.	(Schmitt et al., 2007)

	Duration of heating = 30 min WPI concentration = 1% w/w	• Size of aggregates, intrinsic viscosity increases with reduction in pH. 53 nm (pH =7.0), 53.5 nm (pH = 6.4) and 120.3 nm (pH = 6.0). • Surface hydrophobicity reduced with reduction in pH • Solubility of aggregates increased with reduction in pH and increase in NaCl concentration prior to heating.	
WPI	Heating temperature = 65, 75, 85 °C, pH = 7.0 Duration of heating = 120 min WPI concentration = 10% w/v	• Molecular weight and size of aggregates increased with duration of heating and heating temperature • 40% β-lg remained in native form even after heating for 120 min at 65 °C.	(Kazmierski & Corredig, 2003)
WPI	Heating temperature = 80 °C, pH = 7.0 Duration of heating = 1,3 and 9 hr. WPI concentration = 8,9,10, and 11% w/w	• Aggregate formation is a two-step process. Primary aggregates are formed by covalent bonds between monomers. Final aggregates formed by non-covalent bonds between aggregates. • Intrinsic viscosity increases with WPI concentration. The values remain lower than hydrocolloids even at higher WPI concentration	(Vardhanabhuti & Foegeding, 1999)

$$\ln k_d = \mathrm{n} \ln \alpha + \ln k_{ag} \quad \text{(Equation 3)}$$

For $0 < \alpha < 1$, $\ln \alpha < 0$, unfolding or denaturation step is a rate limiting step.

For $\alpha = 1$, $\ln \alpha = 0$, aggregation is a rate limiting step.

For temperature > 90°C, $\alpha = 1$ as denaturation is assumed to be irreversible. In such a scenario, the rate of aggregation becomes a rate limiting step.

At near neutral pH and for heating temperatures of > 75°C, several studies have noted near complete denaturation of whey proteins within a few minutes which involve both covalent and non-covalent interactions at higher temperatures (de la Fuente et al., 2002; Iametti et al., 1996; Ryan et al., 2013). It was shown this led to the formation of much larger aggregates as compared to heating at lower temperatures.

2.3.2.2 Effect of concentration

A study reported by Zhou et al. (2008) on whey proteins observed aggregation at 35°C at a concentration of 500 g/kg. In this experiment a WPI solution maintained of neutral pH using phosphate buffer solution was kept in an incubator maintained at 35°C for duration of 500 hrs and samples were drawn for analysis at periodic intervals. Whey proteins remain in a non-aggregated steady state for longer duration time unless heated at a temperature of > 65°C. At a constant pH and heating temperature, the size of aggregates is directly proportional to the protein concentration. The size of aggregates and molecular weight of aggregates have shown to increase with protein concentration (Hoffmann & van Mil, 1997; Purwanti et al., 2011; Roefs & De Kruif, 1994). While the rate of denaturation does not depend upon protein concentration, the formation of aggregates or completion of reaction takes longer in the presence of higher protein concentration (Hoffmann & van Mil, 1997; Mahmoudi et al., 2007; Mehalebi et al., 2008). Protein concentration also affects the rheology of resulting dispersion with whey protein aggregates. In an earlier study on polymerised whey protein which were whey protein aggregates prepared by thermal treatment of WPI solution of different concentration (8 to 11% w/w), it was reported that the intrinsic viscosity of aqueous solution with whey protein aggregates increased with protein concentration (Vardhanabhuti & Foegeding, 1999). The possible reason for such increase is due to an increase in volume fraction occupied by proteins. The volume fraction of globular proteins (e.g. whey protein, soy protein and casein) dispersed in aqueous phase has been shown to increase with concentration (Boulet et al., 1998; Roullet et al., 2019).

2.3.2.3 Effect of pH

pH has an impact on denaturation temperature of individual proteins. While denaturation temperature of β-lg is higher at pH 3 compared to pH 6.5 (Arnaudov & de Vries, 2006; deWit & Klarenbeek, 1984). Although the underlying reason remains unclear, a few studies have pointed towards stronger hydrogen bonds in acidic environment, inhibition of thiol or strong repulsive forces between monomers (de la Fuente et al., 2002; Resch et al., 2005). Smaller portions of whey proteins, α-la and BSA, suffer from acid denaturation as the pH is reduced and subsequently, heating temperature has no effect of denaturation of these portions at lower pH (deWit & Klarenbeek, 1984) As β-lg is a dominant component of whey proteins, the denaturation temperature of whey protein is similar to β-lg at a pH ranging from 2 to 8 (Bernal & Jelen, 1985; Pelegrine & Gasparetto, 2005). Solubility of whey protein is dependent on pH of the solution. At pH $\approx$ pI, the solubility of native whey proteins is reduced to approximately 70%. As whey proteins is a mixture of different whey protein molecules, the solubility is depressed for a range of pH 4.4-5.5 (Mehalebi et al., 2008; Pelegrine & Gasparetto, 2005). The presence of large aggregates of whey proteins has been observed without heating at pH close to pI (Ju et al., 1999). As pH is brought to be closer to pI, there is a precipitous decline in critical gel concentration (C_g). A study on the effect of pH on β-lg by Mehalebi et al. (2008), it was shown that C_g of β-lg appeared to gradually increase at pH below or above 4.5 and had much steeper reduction as pH was reduced from 7 to 6.

Besides the difference in the denaturation temperature, the shape of aggregates formed at different pH levels appears to be different. The structural difference observed by a study conducted by Jung et al. (2008) on β-lg noted that for pH = 5.8 (closer to pI), the aggregates were more circular in shape and the aggregates at pH << pI at pH = 2 appeared as fibrillar strands and at pH >> pI at pH = 7 these aggregates were curved in shape. It was also noted that there was a similar difference in the shape of aggregates on heating β-lg by Zúñiga et al. (2010) and WPI by Schmitt et al. (2007) as pH of protein solution was changed from near neutral to pH = 6.0.

As pH is brought closer to the pI of whey proteins, the reduction in repulsive forces leads to formation of larger aggregates. The size of aggregates is possibly also affected by change in the rate of denaturation with change in pH of dispersion. Rate of denaturation has been noted to drop as pH was reduced from 7 to 6 for heating at 65 and 68.5°C by Hoffmann and van Mil (1999) and at 75°C by Verheul et al. (1998). At heating temperature > 80°C, the rate

of denaturation increased as the pH was reduced from near neutral conditions to pH 6 (Zúñiga et al., 2010). Fractal aggregates of a size range of 50 – 400 nm have been synthesized at different protein concentrations but less than 10 % (w/w) (Mehalebi et al., 2008; Nicolai & Durand, 2013; Ryan et al., 2012; Zúñiga et al., 2010) by heating whey proteins at a pH sufficiently away from pI.

2.3.2.4 Effect of ionic strength

Besides pH, the structure of aggregates also depends upon the ionic strength of the solution. The stability of colloidal dispersion of whey protein aggregates depends upon an ionic concentration of the dispersion (Ako et al., 2009). An addition of salt at a constant pH can be viewed as change in pH at a constant ionic concentration (Ryan et al., 2013). The charge screening effect of ionic salts can be compared to that of lowering of pH (Nicolai et al., 2011). The possible change in structure can also be explained by lowering of critical gel concentration at higher ionic strength (Renard & Lefebvre, 1992). It has been observed that at neutral pH, thermal-induced denaturation rate of native β-lg increases in the presence of small amount of salt (Mounsey & O'Kennedy, 2007; Verheul et al., 1998). It was observed that the nature of salt affects denaturation rate, whereby the rate for $CaCl_2$ > NaCl > arginine HCl (Croguennec et al., 2004; Roefs & Peppelman, 2001; Unterhaslberger et al., 2006). $CaCl_2$ which contains divalent cations compared to monovalent cations in NaCl forms proteins aggregates at much lower molar concentration. For pH = 7, on heating, the curved strands of β-lg agglomerate together to form much bigger fractal aggregates or fractal clusters with an increase in NaCl (Baussay et al., 2004; Pouzot et al., 2005). In a study conducted on WPI (7% w/w) when heated to 90°C for 5 min, it was observed that there was an increase in size of aggregates and similar change in structure at NaCl = 54 mM. At NaCl = 108 mM. It was further observed that heated dispersion gelled by Ryan et al. (2012). At pH 6-7, the structure of the aggregates does not change with change in concentration as long as NaCl < 50 mM and concentration < C_g (Phan-Xuan et al., 2013; Pouzot et al., 2004). In an earlier experiment on O/W emulsion stabilised by β-lg, an addition of 150 mM NaCl before the formation of emulsion destabilised the emulsion on heating (Kim et al., 2002). Extensive droplet aggregation observed before heating was due to screening effect of the salt.

Thermally induced whey protein aggregates can also be prepared by dry heating (in absence of water) (**Table 2.5**). Most of the studies on dry heating involve much longer heating time which can be more than several months. Such a high investment in energy and space may not be a viable option to prepare soluble aggregates at a scale with a help of dry heating. Wet heating remains a preferred route for several laboratory investigations and commercial applications. Although dry heating is not relevant in the context of the present work, some important studies pertaining to the subject are highlighted in **Table 2.5**.

2.3.3 Other methods for formation of soluble whey protein aggregates

Denaturation (partial or complete) and aggregation of whey proteins remains at the core of formation of soluble whey protein aggregates. Some of the methods which have been used in the past are thermal, high pressure, ultrasound, pulsed electric field, and ohmic heating. Several studies have proved that α-la is almost resistant to denaturation under high pressure of 500 MPa for a time duration of more than 60 min (Anema et al., 2005; Lopez-Fandino et al., 1996; Messens et al., 1997). While studies did observe some denaturation of α-la at a pressure of 400 MPa, it quickly renatured once the pressure was released (Kobashigawa et al., 1999; Tanaka et al., 1996). In contrast, β-lg undergoes irreversible denaturation and subsequent aggregation at pressures in excess of 150 MPa at pH = 7 (Tanaka et al., 1996) but could be reversed at similar pressure at pH = 3 (Dufour et al., 1994). It can be assumed that behaviour of whey protein consisting of different whey protein molecules is dictated mainly by β-lg (Patel et al., 2005). There have also been some efforts to use gamma, ultraviolet and microwave radiation to prepare self-assembled structures. However, the cost and safety of such methods would be a possible deterrent for commercial use of such process. A few selected studies on formation and evaluation of whey proteins using different methods other than thermal treatment are listed in **Table 2.6**. While efforts persist on different methods, the use of thermal treatment dominates

Table 2.5 Recent studies on the use of dry heating for denaturation and aggregation of whey proteins

PROTEIN	HEATING PARAMETERS (Temp/time/pH)	PROMINENT INFERENCES	REFERENCE
B-lg, WPI & WPH	23 & 45°C, 15 days, pH 7-8, Rh = 11-85%	• Only WPH forms aggregates at lower temperature via covalent bonds. Approx. 20% of these are insoluble.	(Zhou & Labuza, 2007)
WPI	Temp = 4, 20, 40, 60°C Time = 15,30,90,180,270, 360 days pH = Native a_w = 0.23 and 0.36	• For temperature above 40°C, powders showed lactosylation of >20% at both values of a_w. • Insoluble aggregates = 50% for a_w = 0.23 & 60°C and a_w = 0.36 & 40°C and stored at 360 days. • Powder of a_w = 0.36 stored for 180 days at 60°C had 30% better foam stability and 15% higher foam density than WPI stored at less than 40°C for varying time period	(Norwood et al., 2016)
WPI	100°C, 8-24 h, pH = 2.5, 4.6 and 6.5 a_w = 0.23 Solubility of aggregates measured at pH =7, % native monomers deduced by solubility at pH = 4.6	• A maximum of 58% soluble aggregates formed at pH =2.5 on heating for 24h. A concentration of native monomers at 42% achieved at the same conditions • Soluble aggregates in the size range of 50 to 220 nm.	(Gulzar et al., 2011)
WPI	80°C (6 days),100 °C (24 h), 120°C (3h) pH = 2.5, 4.6 and 6.5 a_w = 0.23, 0.32, 0.52 Gelation by heating 10% dry heated powders at 90 °C for 30 min	• Formation of soluble aggregates depends more on a_w and pH compared to temperature/time. Increase in pH reduces native proteins and increases insoluble aggregates. Similar effect is observed with higher a_w. • Gel strength and water holding capacity increases with proportion of % soluble aggregates	(Gulzar et al., 2012)
WPI	100°C (0, 12, 24, 36 h) pH = 3.5, 6.5 and 9.5 a_w = 0.24	• Equal degree of browning for dry heating > 6 h. • The size of particle increased with pH. • pH 3.5 = 97 nm, pH 6.5 = 220 nm, pH 9.5 = 700µm. 100% insoluble aggregates at pH = 9.5 for heating time > 12 h.	(Famelart et al., 2018)

the field and a substantial amount of research in the past decades has provided a better understanding about kinetics of self-assembly, physicochemical properties of structures. There is also a significant body of work focussing on use of these whey protein particles as Pickering emulsion (Chevallier et al., 2016; Dybowska & Krupa-Kozak, 2020) and emulsion gels (Boutin et al., 2007) which would lend a long-term kinetic stability with superior digestion profile and sensory attributes. Its use as an encapsulant of bioactives has attracted a significant attention in the immediate past (Mohammadian et al., 2020). These particles could prove to be used as an emulsifier, and foaming agent as well as a stabiliser and prove to be an important addition to the ingredients for food applications.

2.4 Functional applications of soluble whey protein aggregates

As already mentioned in the earlier sections, "soluble" whey protein aggregates can be regarded as a precursor of whey protein gels. In fact, one of the early mentions of "soluble" whey protein aggregates can be found in the work by McSwiney et al. (1994) on heat-induced gels which noted that protein aggregates are pre-gel particles formed in the dispersion before the formation of an extensive gel network.

2.4.1 Stabilisation of O/W emulsions

Soluble whey protein aggregates possess suitable physical properties (surface hydrophobicity, size of aggregates, foaming stability, intrinsic viscosity, etc.) for its use in O/W emulsions (Brulé & Croguennec 2016; Dybowska, 2011; Schmitt et al., 2007). Heat-induced whey protein aggregates were found to possess higher surface hydrophobicity than native whey proteins on account of its unfolding and aggregation. Surface hydrophobicity of whey protein aggregates has been found higher than native proteins on account of extensive protein unfolding due to thermal treatment (Moro et al., 2001). Intuitively, one may expect that foam stability and emulsifying activity index would also be better for whey protein aggregates, depending on the degree of protein denaturation. However, recent studies have noted that on heating for longer duration at 85°C, foam stability and emulsifying activity index (EAI) was found to be inferior in comparison with native whey proteins (Moro et al., 2013; Moro et al., 2011). It has been noted in the earlier studies that surface hydrophobicity increases with the size of aggregates (Maticorena et al., 2018; Moro et al., 2001; Schmitt et al., 2007). A recent study on aggregates formed by heating β-lg at a pH of 4 or 7 proved that while fibrils (formed at pH = 4) had better foam stabilising property than strands (formed at pH =7), the former proved much inferior emulsifiers (Moro et al., 2013). There have been relatively few studies conducted in the past to understand the characteristics of O/W emulsions and emulsion gels stabilised by whey protein aggregates (**Table 2.7**). While non-heated whey proteins primarily work as an emulsifier and a stabiliser, whey protein aggregates can also be used as a thickening agent. A recent study on O/W emulsion with butterfat as oil phase proved that whey protein aggregates provided better emulsion stability (in terms of creaming rate) compared to native WPI (Zhou et al., 2020). It was also noted that thermal modification of whey protein concentrate provides superior stability against creaming (Dybowska, 2011). However, most of these studies are limited to the use of whey protein aggregates in a narrow size range of 25 – 55 nm.

2.4.2 Rheology of O/W emulsions

A common refrain among several studies on O/W emulsions stabilised by whey protein aggregates is a shear thinning property of emulsions due to depletion flocculation induced by presence of whey protein aggregates in the continuous phase (Boutin et al., 2007; Dybowska, 2011; Dybowska & Krupa-Kozak, 2020). As the size of whey protein aggregates (20 – 40 nm) is much bigger than native whey protein (3 – 7 nm) , it could possibly behave like colloidal sibling – casein (Boutin et al., 2007). Oil droplets in O/W emulsions are separated by presence repulsive forces. This ensures a minimum distance between the droplets. In case of larger colloidal particles in the continuous phase, the distance between droplets could become lesser than diameter of such particles. In such a scenario, the resulting osmotic pressure gradient increases attractive forces between the droplets leading to droplet aggregation. This caused by region separating the droplets being "depleted" of colloidal particles. Such a droplet aggregation or flocculation is termed as "depletion flocculation" or "depletion interaction" (Dickinson, 1992b; Dickinson & Golding, 1997a). It has been noted in the earlier studies that O/W emulsions stabilised by casein behaviour shear thinning on account of depletion flocculation. Depletion flocculation has been noted to occur in the presence of excess protein in the continuous phase (Dickinson & Golding, 1997a, 1997b). Several other process variables like droplet size, protein concentration, homogenisation pressure and oil/fat content could lead to the presence of excess protein in the bulk phase. The size of aggregates has a decisive impact on rheology of dispersions on account of difference in

Table 2.6 Effect of other methods on denaturation and aggregation of whey proteins

PROTEIN	TREATMENT	PARAMETERS	PROMINENT INFERENCES	REFERENCE
WPI	Heating – Conventional (COV) vs Ohmic Heating (OH)	WPI = 3%, pH = 6.8 Temp = 75 - 90°C Time = 30s to 30 min	• % Native protein is higher by 10-20 % for OH vs COV • Order of reaction is close to 1 for OH while it is 1.4 to 1.7 for COV	(Pereira et al., 2011)
WPI	Ohmic heating	WPI = 3% (w/w) Temp = 90°C Time = 5 min pH = 6.8 Electric field = (6 and 12 V cm^{-1})	• Particle size of 100 – 150 nm with solubility of 60 – 70% • Particle size is lower, and solubility is higher for higher electrical voltage • WPI aggregates observed under TEM have a fibrillar structure.	(Pereira et al., 2016)
WPC	High pressure	WPC = 12% (w/v) Pressure = 800 MPa, Time = 0 to 120 min, pH = native (6.65)	• Shear thinning behaviour for time >20 min • Shape of aggregates changes from fine strands (t=20 min) to particulates (t= 40 min) • For t = 20 min, 60 % denaturation, For t = 120, 100% denaturation	(Patel et al., 2005)
WPI, WPC	High pressure	WPI, WPC = 10% (w/w) Pressure = 300 and 600 MPa Time = 5 and 10 min respectively. pH = 6.47 (WPC) and 6.92 (WPI)	• Soluble aggregates at **300 MPa** = 86% (WPC), 88% (WPI) • **600 MPa** = 79% (WPC), 76% (WPI) WPC (600MPa) > Control = WPC (300MPa) WPI (600 MPa) >WPI (300 MPa)>Control • No difference in ability as emulsifier (EAI, ESI) • Superior foaming stability and foam expansion compared to control	(Krešic et al., 2006)
WPI	Pulsed Electric Field (PEF)	WPI = 3% (w/v) Electric field = 12-20 kV cm^{-1} and pulse of 10-30	• Surface hydrophobicity increases with pulses. • PEF does not affect denaturation temperature of WPI	(Xiang et al., 2008)
WPI	PEF and COV	WPI = 1% (w/w), pH= 6.8 Electric field - = 35kVcm^{-1} Time = 19 and 211 µs COV: Temp (time) = 63°C (30 min), 72°C (15 s), 72°C (10 min), 90°C (1 min), and 90°C (10 min)	Emulsion prepared with oil = 10% (w/w), WPI = 0.5% (w/w) Emulsions Properties: • $d_{4,3}$ = 3 µm for all PEF treatments. • Larger size of 4 - 18 µm for temp > 72°C and time >1 min	(Sui et al., 2011)

Table 2.7 List of recent studies on O/W emulsions stabilised by whey protein aggregates and microgels

PROTEIN	TYPE OF PARTICLE	METHOD AND APPLCIATION	PROMINENT INFERENCES	REFERENCE
WPI	Aggregates	**Step 1: Preparation WPI aggregates (WPA)** Heating WPI dispersion (6.25% w/w) at 85°C, 30 min, pH =6.8-7 under stirring **Step 2: Emulsion preparation** WPI/WPA = 0.1, 0.2, 0.5, 1, 2, 4% Milk fat = 20%	• Z-avg diameter of WPA = 20 nm • Creaming rate of WPA was lower than WPI at concentration >2%. $d_{4,3}$ = 1.37 µm for these concentration for both type of emulsions. • Dynamic viscosity of WPA stabilised emulsions is almost twice that of WPI stabilised emulsions	(Zhou et al., 2020)
WPI	Aggregates	**Step 1:** **Preparation of WPA** WPI = 7% (w/w), pH = 6.8. Heated to 90 °C for 10 min **Step 2: Stability with NaCl and heat** WPA = 3% (w/w) NaCl = 0, 54 and 108 mM Heat @ 90°C for 5 min	• Heated dispersion of WPI and WPA did not turn turbid till NaCl = 54 mM • WPA remains stable in NaCl compared to WPI. Sharp increase in plastic viscosity of heat WPI + 108 mM NaCl • Gel formation on heating WPI and WPA and addition of 108 mM NaCl • Z-avg diameter of WPA does not increase after further heat treatment but approx. doubles on addition of 54 mM of NaCl. A similar observation for WPI on addition of 54 mM • Surface hydrophobicity of WPA>WPI	(Ryan et al., 2012)
WPI	Aggregates	**Step 1:** **Preparation of WPA** WPI = 4% and 8%, pH = 7 Heated at 85°C for 30 min **Step 2:** **Preparation of O/W emulsion** Butter oil – 0 to 20% (v/v) Homogenisation pressure = 10, 30, and 12 MPa	• Emulsion droplet size reduced with increase in homogenisation pressure, reduction in oil content and increase in protein concentration. • Droplet size range from 340 nm to 900 nm • Depletion flocculation observed in O/W emulsions stabilised by whey protein aggregates • Apparent viscosity increased with oil content • Oil droplet size did not affect mechanical properties of emulsion gels.	(Boutin et al., 2007)

		Step 3: **Preparation of emulsion gels** @ pH = 4 for O/W emulsions stabilised by 4% and 8% whey protein aggregates in aqueous phase	• Emulsion gels were harder for more protein concentration	
WPC	Aggregates	**Step 1:** **Preparation of WPA** WPC = 3%, pH = 7 Heated at 60, 70, 80, 90 and 95°C for 5 min **Step 2:** **Preparation of O/W emulsion** WPC and WPA = 3% w/w Rapeseed oil = 30% w/w Homogenisation pressure = 10 MPa	• Particle size of whey protein aggregates increased from 1281 at 80°C to 1718 nm at 95°C. • No aggregation noted for < 80°C. • All emulsions were bimodal in nature • Stability of emulsions measured from phase separation on centrifuging was higher for emulsions stabilised by WPA.	(Dybowska, 2011)
WPC	Aggregates	**Step 1:** **Preparation of WPA** WPC = 3%, pH = 6.3 Heated at 60, 70, 80, and 90°C for 5 min **Step 2:** **Preparation of O/W emulsions (HO)** WPC = 3% Rapeseed oil = 30% w/w O/W Emulsions heated 60, 70, 80, and 90°C for 5 min **Step 3:** **Preparation of O/W emulsions (WPO)** WPA = 3% w/w, Rapeseed oil = 30% w/w	• Droplet size of WPO > HO • Stability of emulsions measured from phase separation on centrifuging was higher for WPO. • HO for heating temperatures > 80°C showed aggregation of whey proteins at the interface. • Both HO and WPO displayed shear thinning properties on account depletion flocculation	(Dybowska & Krupa-Kozak, 2020)

β – lg	Aggregates	**Step 1:** Preparation of β-lg aggregates (BA) B-lg = 0.5% w/v, pH = 7 Heated at 69 °C for 30 and 45 min **Step 2:** **Preparation of O/W emulsion** B-lg and BA = 0.5% w/w Rapeseed oil = 60% w/w Homogenisation pressure = 50 MPa	• Size of aggregates (25 – 40 nm) did not change with duration of heating. • Surface hydrophobicity and surface load was higher for longer duration of heating. Surface hydrophobicity and surface load of BA > β-lg. • Droplet size of emulsions stabilised by β-lg was lower than emulsions stabilised by BA. • Emulsions stabilised by BA were soft solids while emulsions stabilised by β-lg remained fluid.	(Knudsen et al., 2008)
WPI	Microgel	Heat induced aggregates WPI = 0.1-6% Temp =25 - 85°C, time = 30 min **Step 1: Premix with CaCl2** WPI = 1% (w/w), $CaCl_2$- 0-10 mM, pH = 7. **Step 2**: Heating – 25 - 85°C for time = 30 min to create WP microgel (WPM)	• WPI aggregates of size range 30-200 nm formed for temp > 65°C. Size distribution is bimodal for higher temperature and concentration >1%. • Z-avg diameter = 178 nm for 5% at 85°C • At 10 mM $CaCl_2$ – precipitation at 75 °C and gelation at 85°C. WPM of Z-avg diameter = 255 for 5 mM. The size of microgels increases with temp and concentration of $CaCl_2$.	(Ni et al., 2015)

WPI	Microgel	**Step 1: WPI gel** WPI = 10% (w/w), Heating at 90°C for 30 min at pH =7, ionic strength = 0. **Step 2: Gel Homogenisation of** WPI microgel (WPM) dispersion formed with 5% (w/w). Emulsion prepared with oil = 20%, WPM = 1%. Emulsions heated to 90°C for 30 min **Step 3:** In vitro digestion study in gastric and intestinal phase for both heated and non-heated emulsions	• Z-avg of WPM = 311 nm, • d_{43} = 43 µm for both heated and non-heated emulsions. Surface load increased from 14 to 23 mg/m^2 on heating. % Free fatty acid released for heated and non-heated WPM emulsions was slightly lower than WPI emulsions (42% vs 46%)	(Sarkar et al., 2016)
WPI	Aggregates and Microgels	**Step 1: Preparation of WPA** Heating WPI dispersion (8% w/w) at 80°C, 120 min, pH =7 **Preparation of WPM** WPI = 5% (w/w), pH = 5.8. Heated to 85°C for 60 min **Step 2:** **Preparation of acid milk gels** Milk fat = 3.5, 10% WPI, WPA, WPM – 1.5% (total protein = 5%) GDL based reduction to pH =4.6	• Z-avg diameter of WPA = 68 nm, WPM = 458 nm • Gel stiffness (G') for WPI and WPA increases with protein concentration • Gel stiffness does not change with WPM • Gel stiffness → WPI>WPA>WPM = control Gels with 1% WPA does not show any whey separation after 7 and 14 days. Approx. 25% whey separation for WPI and WPM	(Gélébart et al., 2019)

physical properties like surface viscosity and intrinsic viscosity (Eissa et al., 2014; Schmitt et al., 2007; Vardhanabhuti & Foegeding, 1999). For flocculated emulsions with whey protein aggregates, the rheology of emulsions would be affected by adsorbed as well as non-adsorbed whey protein aggregates. For non-flocculated emulsions stabilised by non-heated whey proteins, apparent viscosity of emulsions has been shown to increase with oil content (Bullard et al., 2009; Pal, 2000). The rheology of such flocculated emulsions has been shown to be affected by change in oil content (Boutin et al., 2007). However, there have been very few studies to understand the characteristics of emulsions formed by whey protein aggregates at varying oil content and its thermal stability.

2.4.3 Effect of thermal treatment

Heat treatments, such as thermisation, sterilisation, pasteurisation and UHT, have been used to preserve the shelf life of food products (Deeth & Lewis, 2017). It has been noted in the previous studies that soluble whey protein aggregates have a better thermal stability compared to native whey proteins (Monahan et al., 1996; Ryan et al., 2012). However, favourable thermal behaviour of whey protein aggregates is limited to heating temperatures of < 95°C (Dybowska, 2011; Dybowska & Krupa-Kozak, 2020). For temperatures > 120°C, emulsions stabilised by whey protein aggregates suffered extensive droplet aggregation. Emulsions stabilised by whey protein aggregates in conjunction with casein have been found to have substantially improved heat stability at temperature > 120°C for concentration > 4% w/w (combined protein content of whey protein and casein) (Chevallier et al., 2016). There have been several studies in the past on thermal treatment of O/W emulsions stabilised by whey proteins (Euston & Hirst, 1999; Monahan et al., 1996; Monahan et al., 1997; Ye, 2008) and a few on protein rich emulsions (Liang et al., 2013). However, the thermal stability of O/W emulsions stabilised by whey protein aggregates has not been well studied; both in protein-poor and protein rich emulsions.

2.4.4 Effect of pH

Heat-induced whey protein aggregates have been extensively studied in the field of cold set gels. Cold set whey protein gel is a two-step process. In the step one, whey protein solution is heated at a sufficient time above its denaturation temperature of 75°C to form a stable dispersion of soluble whey protein aggregates. In the second step the repulsive forces between aggregates are reduced by acidification or by addition of cations (mineral salt) (Alting et al., 2000; Bryant & McClements, 1998; Doi, 1993). Cold set process allows the formation of whey protein gels at protein concentrations < C_g. The second step in the process or “cold-setting” takes place at ambient temperature unlike heat induced whey protein gels. The formation of cold set gels has been found to be extremely important when one uses protein gels to encapsulate natural bioactives of heat sensitive ingredients. The structure and shape of whey protein aggregates formed in step one has a significant influence on the mechanical and textural properties of cold-set gels (Alting et al., 2003).It is equally worth noting that there is no evidence for change in isoelectric point of whey proteins on thermal treatment. Possibly, O/W emulsions stabilised by soluble whey protein aggregates may also suffer destabilisation as observed in those stabilised by non-heated proteins. In fact, a range of studies in the field of emulsion gels and emulsion filled gels containing whey protein aggregates have been induced by change in pH (Boutin et al., 2007; Rosa et al., 2006; Sok Line et al., 2005).

2.4.5 Effect of ionic strength

O/W emulsions stabilised by native whey protein suffer extensive droplet aggregation on addition of mineral salts. It has been found in the previous studies that addition of >150 mM of NaCl and > 3 mM of $CaCl_2$ leads to extensive flocculation due to droplet aggregation (Demetriades et al., 1997; Kulmyrzaev et al., 2000; Ye & Singh, 2000). However, whey protein aggregates have been shown to have improved stability to ionic conditions (NaCl) and could thus be used effectively in beverages (Ryan & Foegeding, 2015). The study could not establish the underlying reasons for the improved stability of soluble whey protein aggregates against salts. There is no evidence from a wider search of literature that whey protein aggregates have a better stability to salt than the native whey protein. In fact, a number of studies in the past have noted the formation of cold-set gels on addition of salts to thermally treated whey protein solution (Barbut & Foegeding, 1993; Bryant & McClements, 2000; Hongsprabhas & Barbut, 1997). There have also been a few studies related to the formation of emulsion gels on addition of salts (Kharlamova et al., 2018; Ye & Taylor, 2009).

2.4.6 Other uses

A few recent studies on the use of whey protein aggregates as encapsulating agent for bioactives have observed that tortuous structures or interfaces created by whey protein aggregates are less prone to rapid proteolysis in gastro-intestinal tract (Guo et al., 2014; Singh et al., 2015). In a recent study, whey protein nanofibrils were shown to improve the bioavailability of curcumin (Mohammadian et al., 2019) and iron (Shen et al., 2017) by slowing their release in the gastro-intestinal tract. The demand for high protein and reduced fat products has been rising steadily in the recent years. The present experimental work is an endeavour to create a toolkit for the preparation of O/W emulsions with varied physical and chemical properties to suit the needs of a real-world food formulator by forming and using soluble whey protein aggregates.

2.5 Literature review conclusions

Although there have been several studies in the recent years to understand the characteristics of whey protein aggregates, the formation of soluble whey protein aggregates has not been extensively studied at higher whey protein concentrations. The physical properties of soluble whey protein aggregates have been shown to be affected by heating temperature, duration of heating and pH of solution. The present study on the formation of soluble whey protein aggregates at higher WPI concentration is an attempt to address this knowledge gap (Chapter 3). Soluble whey protein aggregates have emerged as an important functional ingredient in the recent times on account of its favourable stabilising and rheological properties. These properties can be used to prepare high protein fluid emulsions with desirable structural and textural properties as well as high protein beverages. Most of these studies have been conducted at whey protein concentration less than 4%. It seems that there have been no studies which have explored the effect of particle size of whey protein aggregates on the physical and chemical characteristics of O/W emulsions. In fact, there have been even fewer studies in the realm of protein rich O/W emulsions. The current experimental work was undertaken to understand to improve the understanding of effect of particle size of soluble whey protein aggregates on the formation and stability of O/W emulsions (Chapter 4). Oil content is known to affect the rheology of O/W emulsions. An experimental work was undertaken to understand the combined effect of oil content and particle size of whey protein aggregates on the rheology and stability of O/W emulsions (Chapter 5).

Chapter 3. Formation and characterisation of soluble whey protein aggregates by heat treatment

3.1 Abstract

Soluble whey protein aggregates were formed from commercially available whey protein isolate (WPI) by thermal treatment by altering three process parameters, such as heating temperature (68, 75 and 85°C), duration of heating (up to 60 min) and pH of whey protein solution (6.9,6.4 and 6.0). Higher duration of heating (60 min) was chosen for lower temperature of 68 °C and lower heating duration (25 min) was chosen for higher temperatures. WPI solution at a concentration of 8% (w/w) and negligible ionic strength was used for the experimental work. Soluble whey protein aggregates were characterised for their particle size, ζ–potential and yield. The characteristics of soluble whey protein aggregates were significantly affected by pH of the solution for all heating temperatures. A significant effect of duration of heating was observed on the particle size of aggregates only at heating temperature of 68°C and at pH = 6.9. While the particle size of aggregates increased, the yield of soluble aggregates decreased as the pH of solution prior to heating. A sharp reduction in the yield of soluble whey protein aggregates to 34.5% was observed for heating at 75°C for 25 min and at pH = 6.0. This was unlike much higher yields of 89.1% and 78.5% obtained by heating at 68°C and 85°C respectively with solution maintained at pH = 6.0 prior to heating. The experimental study was able to establish process parameters necessary to form soluble whey protein aggregates with varying particle size and yield.

3.2 Introduction

The consumption of protein has been on the rise on account of several nutritional benefits in order to achieve weight control, combat lifestyle ailments like hypertension and cardio-vascular diseases and improve post-workout muscle fatigue (Binns et al., 2015; Borreani et al., 2017; Layman et al., 2009). By virtue of its superior nutritional profile owing to a balanced amino acid content and a high value of essential amino acids and digestible indispensable amino acid score, bovine whey proteins are an important ingredient in several nutritional and sports supplements and infant formula (Berrazaga et al., 2019; Deeth & Bansal, 2019; Hoffman & Falvo, 2004; Norton et al., 2021; Smithers, 2008; Wagner et al., 2020). Whey proteins also serve several non-nutritional functions in food (Foegeding & Davis, 2011). Whey proteins have been widely used in food formulations due to desirable properties like solubility (beverages), emulsification (coffee whiteners, dairy desserts), foaming (mousse, shakes), water binding (meat sausages, soups) and gelation (meats, puddings) (Boland, 2011; Singh et al., 2014).

Heat induced denaturation and aggregation of whey proteins have been studied extensively by several researchers for its relevance in dairy processing (fouling of heat exchangers), and storage stability of milk and other commercial products (De Wit, 1990; Deeth, 2020; Harwalkar, 1992). A controlled heat-induced aggregation has been used to expand functionality of whey proteins to prepare cold set gels (Barbut & Foegeding, 1993; Bryant & McClements, 1998), fat replacer (Kew et al., 2020; Torres et al., 2018; Urgu et al., 2019) and emulsion stabiliser (Boutin et al., 2007; Dybowska, 2011; Sarkar et al., 2016). Some of the prominent types of particles/aggregates prepared in recent times by thermal denaturation and aggregation of whey proteins include microparticulated whey proteins (MWPs), nanoparticles, microgels, soluble whey protein aggregates (Kew et al., 2020). In the context of modern demand for reduced fat products (David J. McClements, 2015), the use of whey protein particle as a "fat replacer" is of a significant importance (Famelart et al., 2015; Kew et al., 2020).

An important domain of "soluble" whey protein aggregates has emerged in pursuit of whey protein structures or particles with lower particle size than MWPs. An initial use of the term "soluble" protein aggregates was introduced in a study conducted by McSwiney et al. (1994) in which gels were formed by heating β-lactoglobulin (β–lg). "Soluble" whey protein aggregates have been characterised as an intermediate structure before protein forms an extensive gel network or insoluble precipitate based on process condition. Solubility of whey protein aggregates could be defined both as a measure of denaturation when measured at pH ≈ pI (5.1 or 4.6) (Gough & Jenness, 1962; Ryan et al., 2012; Zúñiga et al., 2010) or as a measure of colloidal stability in an aqueous medium without changing pH of the dispersion or solution (Donato et al., 2009; Gulzar et al., 2012; Hudson et al., 2000; Loveday, 2016). Soluble whey protein aggregates with a sub-micron particle size have been prepared in some studies by thermally induced denaturation and aggregation of whey proteins or β–lg (Roefs & De Kruif, 1994; Ryan et al., 2012; Schmitt et al., 2007; Vardhanabhuti & Foegeding, 1999; Zúñiga et al., 2010). In order to enable its use as a fat replacer, soluble whey protein aggregates should not only work as an effective emulsifier but also act as a rheology modifier or a thickener. Some of the relevant properties like surface hydrophobicity, emulsifying activity index and intrinsic viscosity have been shown to be influenced by particle size (Moro et al., 2013; Schmitt et al., 2007; Zúñiga et al., 2010) and concentration of aggregates (Boutin et al., 2007; Vardhanabhuti & Foegeding, 1999).

The understanding of underlying kinetics of the denaturation and aggregation process and influential process parameters has improved due to a significant body of research work in the past few decades on denaturation and aggregation of whey proteins (Croguennec et al., 2004; Kazmierski & Corredig, 2003; Roefs & De Kruif, 1994; Schmitt et al., 2007; Tolkach & Kulozik, 2007). There have also been some excellent review articles on this subject (Famelart et al., 2015; Nicolai et al., 2011; Nicolai & Durand, 2013). Formation of soluble whey protein aggregates can be considered as a series of steps as proteins are transformed into soluble aggregates by molecular interactions such as covalent and non-covalent bonds (Croguennec et al., 2004; Surroca et al., 2002; Tolkach & Kulozik, 2007).

There have been several studies in the past to understand the influence of protein concentration, temperature, pH, and ionic strength on important physical and chemical characteristics of soluble whey protein aggregates. The size of aggregates and proportion of soluble aggregates or insoluble larger aggregates have been shown to be influenced by protein concentration (Vardhanabhuti & Foegeding, 1999), heat treatment temperature (Britten & Giroux, 2001; Kazmierski & Corredig, 2003) and pH of protein solution (Schmitt et al., 2007; Zúñiga et al., 2010). However, most of the recent work on formation of soluble whey protein aggregates have been undertaken at moderate concentrations of (< 6% w/w) of whey protein or β-lg (Chevallier et al., 2016; Mehalebi et al., 2008; Nicolai et al., 2011; Ryan et al., 2012; Schmitt et al., 2007; Zúñiga et al., 2010). There have been fewer studies undertaken at higher concentrations of whey protein/β-lg (Boutin et al., 2007; Vardhanabhuti & Foegeding, 1999). Much fewer of them have studied the characteristics of soluble whey protein aggregates at pH 6.0. There have been no studies on the formation of soluble whey protein aggregates at a temperature close to denaturation temperature of β-lg (75°C) at pH <7. The aggregates formed at lower temperature of 68°C and pH < 7 also have not been studied extensively. Kinetics of denaturation and aggregation at lower temperature (65°C to 68.5°C) and a wider pH range (6.0 to 8.0) have been studied in the past for β-lg but the characteristics of soluble protein aggregates (e.g. particle size, solubility, etc.) have not been studied for these process parameters (Hoffmann & van Mil, 1997; Iametti et al., 1996; Verheul et al., 1998).

Therefore, the objective of this study was to study the formation of soluble whey protein aggregates at WPI concentration of 8% w/w and a wider temperature range (68, 75 and 85°C) and pH values of 6.9 (close to native), 6.4, and 6.0. The temperatures have been chosen in order to capture both covalent and non-covalent interactions in formation of soluble whey protein aggregates as these have shown to affect important characteristics like particle size and solubility (Croguennec et al., 2004; Ryan et al., 2012; Schmitt et al., 2007; Vardhanabhuti & Foegeding, 1999). In this study, the term "soluble whey protein aggregates" means whey protein aggregates soluble in water at the pH of the dispersion. It does not depict the degree of denaturation as used in other earlier works on this topic ((Ryan & Foegeding, 2015; Schmitt et al., 2007). The experimental work was conducted using a combination of process parameters in order to achieve the highest yield of water-soluble whey protein aggregates. The physical properties of the soluble whey protein aggregates measured during the process are expected to have a significant influence in its use as an emulsifier, stabiliser and foaming agent in preparation of high protein, reduced fat emulsions.

3.3 Materials and Methods

3.3.1 Materials

WPI (Surestart WPI 895) was obtained from Fonterra Co-operative Ltd. (Auckland, New Zealand). According to the product specification provided by the manufacturer, the WPI contained 93.54% of protein as shown in **Table 3.1**. All other chemicals used were of analytical grade.

Table 3.1 Key physical and chemical properties of Surestart WPI 895

COMPONENT	VALUE (% w/w)
Protein (6.38 x N) as is	93.54
Fat	0.35
Moisture	4.58
Lactose monohydrate	0.2
pH @ 5% TS 20°C	6.9
Calcium (ppm)	40
Sodium (ppm)	623

Source: *WPI 895 COA, COA number - 85405089-10-1*

3.3.2 Preparation of whey protein solution

A stock solution of WPI (20% w/w) was prepared by slowly dissolving WPI in de-ionised water and added with sodium azide (0.02% w/w) to avoid microbial contamination. The WPI solution was stirred gently for 24 hr at 20°C for complete dissolution and hydration. The stock solution was then centrifuged at 20,000 g for 40 min at 4°C to remove any insoluble whey protein aggregates and further diluted to prepare a WPI solution of 8% w/w. The native pH of the WPI solution (8% w/w) prepared was measured to be 6.9, confirming the data provided in the product specification from the manufacturer (Table 3.1). The WPI solution (8% w/w) was also adjusted to pH 6.4 and 6.0 by using HCl solutions. The WPI solutions with different pH levels (6.0, 6.4 and 6.9) were stored at 4°C until use.

3.3.3 Preparation of soluble whey protein aggregates

To prepare whey protein soluble aggregates, a 15 ml of 8% WPI solution was first transferred into a series of glass test tubes with flat screw stopper. The tubes containing the WPI solution were placed in a water bath for heat treatment at three different temperatures (68, 75 and

85°C) for different times as shown in **Table 3.2.** The time taken for the 15 ml of WPI solution in the test tubes to reach the desired temperature was 68 sec (68°C), 95 sec (75°C), and 120 sec (85°C). The relevant sample codes with heating temperature, time and solution pH are also shown in Table 3.2.

On removal from the water bath, individual test tubes were quickly cooled under flowing cold water and kept in ice bath (10°C) for 20 min and stored at 4°C until further analyses. Non-heated WPI solution was considered as the control sample. The experiment for heat treatment was carried at least in duplicate.

Table 3.2 Experimental design of formation of soluble whey protein aggregates

Sample code	**WPI (% w/w)**	**Eff. protein conc. (% w/w)***	**pH**	**Temp (°C)**	**Heating time (min)**
pH6.9/T68	8	7.5	6.9	68	5,10,15,20,25, 30,35,40,45,50, 55,60
pH6.4/T68	8	7.5	6.4	68	
pH6.0/T68	8	7.5	6.0	68	
pH6.9/T75	8	7.5	6.9	75	1, 2.5, 4, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25
pH6.4/T75	8	7.5	6.4	75	
pH 6.0/T75	8	7.5	6.0	75	
pH6.9/T85	8	7.5	6.9	85	
pH6.4/T85	8	7.5	6.4	85	
pH 6.0/T85	8	7.5	6.0	85	

** Effective protein concentration which is the actual whey protein concentration calculated based on the WPI containing 93.54%.*

3.3.4 Determination of yield of soluble whey protein aggregates

Heat treated whey protein solutions were centrifuged at 12,000 g for 40 min at 4°C to remove any insoluble aggregates formed after the thermal treatment. The supernatants were collected and analysed for the concentration of whey protein by measuring their absorbance at 280 nm using a UV-Visible absorbance spectroscopy (Shimadzu UV 1600) (Kuipers & Gruppen, 2007; Rukke et al., 2010). Proteins display a characteristic absorption in the ultraviolet range at 280 nm (Aitken & Learmonth, 2009; Lüthi-Peng & Puhan, 1999). This is due to the presence of aromatic amino acids; predominantly due to tyrosine and tryptophan

(Britten & Giroux, 2001; Hudson et al., 2000; Noble, 2014; Shimada & Cheftel, 1988). The composition of aromatic amino acids in a typical β-lactoglobulin, α-lactalbumin and WPI (WPI 895) is provided in **Table 3.3**.

The supernatant withdrawn from each protein solution was diluted at pre-determined values with de-ionised water prior to its protein content measurement. The supernatant withdrawn from each protein solution was diluted at pre-determined values with de-ionised water prior to its protein content measurement. The diluted samples were transferred to glass cuvettes and absorbance was measured at 280 nm. A standard curve with a correlation coefficient value (R^2) of 0.97 created using a series of non-heated WPI and heated WPI (85°C for 30 min) solutions of known protein concentrations was used to calculate the concentration of whey protein in the supernatant samples. The supernatant withdrawn from each WPI solution was diluted at pre-determined values with deionised water prior to its absorbance measurement. The measured absorbance values were converted to % yield of soluble whey protein aggregates based on the created standard curve.

It should be noted that heated whey protein solutions would contain different forms of whey protein (e.g. native, soluble and insoluble aggregates) depending on the degree of heat treatment applied. As mentioned above, heat treated solutions were centrifuged to remove insoluble whey protein aggregates. Whey protein aggregates present in the supernatant without precipitation were defined as “soluble” whey protein aggregates.

Table 3.3 Aromatic amino acid content in whey proteins

AROMATIC AMINO ACID	β-lactoglobulin	α–lactalbumin	WPI 895
Phenylalanine	3.2	4.2	3.0
Histidine	1.5	2.9	1.7
Tryptophan	2.0	5.3	1.3
Tyrosine	3.6	4.6	3.4

Source: (Etzel, 2004), Values expressed as % w/w of protein.

3.3.5 Determination of particle size of soluble whey protein aggregates

The particle size of soluble protein aggregates present in the supernatants was measured using ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, England) based on dynamic light scattering (DLS). The DLS technique is based on the measurement of Brownian motion of the particles (Analytical, 2013). In a sufficient dilute solution, the speed of Brownian motion of the particle is based on size of the particles. The correlation functions built-in equipment software provides particle size distribution data. While the fundamental size data from the equipment is based on intensity, it is converted to volume and number distribution based on Mie theory. ZetaSizer Nano ZS is equipped with helium/neon laser with a wavelength of 632.8 nm and uses a backscattering angle of 173^{o} for the measurement of particle size. The refractive index (RI) for protein aggregates and dispersion medium (water) was 1.456 and 1.33, respectively (Analytical, 2013). The supernatants from the protein solutions were diluted at a ratio of 1:100 with de-ionised water before measurement of particle size. The particle size was expressed as Z-average diameter/hydrodynamic diameter (based on intensity distribution). The particle size distribution of samples was based on volume mean diameter.

3.3.6 Determination of ζ-potential of soluble whey protein aggregates

The zeta (ζ)-potential of soluble protein aggregates in the supernatants was also measured using ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, England) which was based on electrophoretic mobility. The ζ-potential was analysed by using disposable cuvettes with folded capillary (DTS1070). Under an electric field, the particles of protein are attracted towards an opposite electrode. This movement is termed as electrophoresis. The equipment calculates the ζ-potential by measurement of electrophoretic mobility (Analytical, 2013). In this study, the supernatants withdrawn from the protein solutions were diluted to a ratio of 1:200 with de-ionised water before measurement to avoid multiple scattering.

3.3.7 Statistical analysis

All the measurements were conducted at least in duplicate, and the results are presented as mean values ± standard deviation. A one-way ANOVA was carried to determine a significant effect of treatment effect at a level of significance ($P \leq 0.05$).

3.4 Results and Discussion

Heat-induced denaturation and aggregation of WPI solution was studied for the formation of soluble whey protein aggregates which were defined as those proteins remaining soluble (or dispersible) in the supernatant of heat treated WPI solutions without precipitation after centrifugation. The soluble protein aggregates were analysed for three key physical properties, such as particle size or hydrodynamic diameter, ζ-potential and the content (yield) of soluble protein aggregates. In this study, the three treatment variables, such as heat treatment temperature, heating time and pH of WPI solution, were analysed for their effects on the formation and properties of soluble whey protein aggregates. The key properties for soluble whey protein aggregates formed under the different conditions of temperatures (68, 75 and 85°C), heat treatment times (5-60 min at 68ºC and 1-25 min at 75 and 85°C) and pH values (6.0, 6.4, and 6.9) are summarised in **Tables 3.4, 3.5 and 3.6**. The WPI (Surestart 895) chosen for this experimental work had a negligible amount of calcium and sodium (**Table 3.1**). It was thus assumed that its ionic strength had no significant relevance in the formation of protein aggregates.

3.4.1 Yield of soluble whey protein aggregates (SWPA)

Solubility of whey protein aggregates or proportion of soluble whey protein aggregates (SWPA) was observed to be significantly influenced by pH of WPI solution ($P \leq 0.05$) for all heating temperatures of 68°C **(Fig 3.1A)**, 75°C **(Fig 3.1B)** and 85°C **(Fig 3.1C)**. A higher value (%) of SWPA means a lower proportion of larger insoluble aggregates formed in the heat-treated WPI solution. This trend confirms with the proportion of larger size of aggregates observed in the earlier studies by Schmitt et al. (2007) for pH range of 6.0 to 7.0 and by Zúñiga et al. (2010) for 6.0 to 6.8 on heat induced denaturation of WPI (1% w/w) and β-lg (5% w/w). As the pH of solution is reduced, the proportion of larger insoluble aggregates increases due to reduction in repulsive forces between the protein molecules which promotes protein aggregation. The observed increase in the greater amount of insoluble protein aggregates can also be explained by an increase in molecular weight of aggregates of whey proteins and β-lg reported in earlier studies by de la Fuente et al. (2002) and Mehalebi et al. (2008).

Table 3.4 Key physical properties of soluble whey protein aggregates formed on thermal treatment of 8% (w/w) WPI solutions (pH 6.0, 6.4 and 6.9) at temperature = **68 °C for** different heating times.

		pH 6.0			**pH 6.4**			**pH 6.9**		
SN.	**Heating time (min)**	**Z-avg dia (nm)**	**ζ-potential (mV)**	**Soluble WPI aggregates (%)**	**Z-avg dia (nm)**	**ζ-potential (mV)**	**Soluble WPI aggregates (%)**	**Z-avg dia (nm)**	**ζ-potential (mV)**	**Soluble WPI aggregates (%)**
1	**native**	**12.3 ± 1.8**	**-39.3**	**N/A**	**9.8 ± 1.3**	**-41.2**	**N/A**	**6.8 ± 1.5**	**-42.3**	**N/A**
2	5	76.3 ± 0.2	-31.8 ± 0.9	93.2 ± 0.5	52.9 ± 1.8	-37.2 ± 0.7	92.7 ± 0.1	16.2 ± 1.0	-32.5 ± 1.0	95.1 ± 0.3
3	10	76.0 ± 0.0	-30.8 ± 0.7	92.6 ± 0.7	49.1 ± 1.6	-33.4 ± 3.2	92.1 ± 0.2	13.7 ± 0.8	-31.4 ± 1.4	94.8 ± 0.2
4	15	80.6 ± 0.1	-32.3 ± 1.5	93.0 ± 1.2	43.2 ± 2.6	-33.5 ± 1.5	92.4 ± 0.1	17.1 ± 1.2	-29.8 ± 1.4	94.6 ± 0.3
5	20	93.3 ± 2.8	-33.8 ± 1.1	90.7 ± 0.8	39.5 ± 0.4	-31.9 ± 0.3	91.8 ± 0.1	36.2 ± 1.8	-31.5 ± 1.0	94.3 ± 0.8
6	**25**	**104.7 ± 0.8**	**-27.5 ± 0.2**	**90.9 ± 1.2**	**34.7 ± 0.1**	**-33.3 ± 1.1**	**91.6 ± 0.9**	**61.6 ± 0.5**	**-33.1 ± 0.4**	**93.8 ± 0.2**
7	30	113.7 ± 1.6	-27.9 ± 0.5	90.9 ± 0.3	41.4 ± 0.9	-31.9 ± 1.0	91.3 ± 0.9	51.7 ± 1.4	-31.8 ± 0.5	93.4 ± 0.2
8	35	107.8 ± 0.5	-28.5 ± 1.3	90.4 ± 0.8	42.0 ± 1.1	-32.6 ± 0.8	90.9 ± 0.1	51.5 ± 2.2	-31.8 ± 0.4	93.8 ± 0.2
9	40	122.8 ± 1.9	-29.1 ± 0.8	90.8 ± 1.7	36.7 ± 0.4	-33.7 ± 3.7	90.9 ± 0.2	47.1 ± 0.0	-30.5 ± 1.1	93.3 ± 0.6
10	45	121.7 ± 0.5	-26.8 ± 1.0	91.6 ± 0.7	37.9 ± 0.3	-29.6 ± 0.5	90.8 ± 0.1	45.9 ± 0.6	-32.2 ± 0.8	93.5 ± 0.0
11	50	122.3 ± 0.1	-28.8 ± 0.6	90.6 ± 0.1	37.9 ± 0.2	-28.3 ± 0.7	90.7 ± 0.3	48.3 ± 1.8	-32.7 ± 0.7	93.9 ± 0.3
12	55	118.1 ± 0.8	-28.2 ± 0.5	88.8 ± 0.5	65.3 ± 0.7	-27.5 ± 0.6	90.4 ± 0.2	47.5 ± 2.2	-30.5 ± 0.3	94.1 ± 0.0
13	**60**	**125.1 ± 1.0**	**-27.9 ± 0.7**	**89.1 ± 0.5**	**78.0 ± 3.0**	**-27.0 ± 1.4**	**90.0 ± 0.1**	**46.2 ± 1.6**	**-29.1 ± 1.4**	**94.0 ± 0.2**

Table 3.5 Key physical properties of soluble whey protein aggregates formed on thermal treatment of 8% (w/w) WPI solutions (pH 6.0, 6.4 and 6.9) at **75 °C** for different heating times. *(Letters a,b,c,d,e,f,g and h means statistically different* (P ≤ 0.05).

		pH 6.0			**pH 6.4**			**pH 6.9**		
SN.	**Heating time (min)**	**Z-avg dia (nm)**	**ζ-potential (mV)**	**Soluble WPI aggregates (%)**	**Z-avg dia (nm)**	**ζ-potential (mV)**	**Soluble WPI aggregates (%)**	**Z-avg dia (nm)**	**ζ-potential (mV)**	**Soluble WPI aggregates (%)**
1	native	**12.3** ± **1.8**	**-39.3**	**N/A**	9.8 ± 1.3	**-41.2**	**N/A**	6.8 ± 1.5	**-42.3**	**N/A**
2	1	70.5 ± 0.4	-28.7 ± 0.4	91.2 ± 0.1[a]	51.9 ± 0.5	-33.5 ± 2.2	93.5 ± 0.1[g]	41.7 ± 0.2	-34.5 ± 0.0	93.5 ± 0.1
3	2.5	80.0 ± 2.1	-27.8 ± 0.2	91.2 ± 0.0[a]	51.7 ± 0.4	-34.2 ± 1.1	91.8 ± 2.0[g]	39.6 ± 1.6	-35.1 ± 0.4	93.4 ± 0.1
4	4	94.5 ± 2.7	-28.0 ± 1.1	87.4 ± 1.8[b]	50.8 ± 0.1	-32.2 ± 2.9	93.3 ± 0.1[g]	67.0 ± 0.8	-34.2 ± 0.1	93.2 ± 0.5
5	5	87.8 ± 1.5	-23.0 ± 0.7	85.4 ± 0.5[b]	53.2 ± 0.4	-30.2 ± 0.3	93.1 ± 0.1[g]	40.9 ± 0.0	-34.7 ± 0.1	93.2 ± 0.1
6	7.5	84.8 ± 1.2	-22.7 ± 0.6	79.0 ± 1.1[c]	45.4 ± 0.1	-29.9 ± 1.2	93.3 ± 0.1[g]	40.7 ± 0.2	-34.7 ± 0.4	93.3 ± 0.2
7	10	110.3 ± 4.0	-24.9 ± 1.0	77.7 ± 0.4[c]	43.1 ± 0.5	-29.9 ± 1.0	91.4 ± 2.8[g]	50.6 ± 0.4	-35.0 ± 0.1	93.3 ± 0.2
8	12.5	102.2 ± 2.4	-25.1 ± 2.9	74.7 ± 0.9[c]	47.0 ± 1.4	-32.3 ± 1.2	93.3 ± 0.1[g]	54.0 ± 0.6	-34.3 ± 0.0	92.3 ± 2.1
9	15	106.2 ± 0.5	-22.5 ± 0.2	55.8 ± 0.4[d]	53.4 ± 0.5	-31.2 ± 1.2	91.4 ± 0.2[g]	43.5 ± 0.4	-34.7 ± 0.5	92.8 ± 0.4
10	17.5	109.5 ± 1.5	-25.0 ± 0.3	48.4 ± 2.8[d]	47.1 ± 0.6	-31.4 ± 0.6	89.7 ± 0.7[g]	39.6 ± 0.8	-34.8 ± 0.1	92.6 ± 0.1
11	20	145.1 ± 0.8	-22.8 ± 0.1	41.8 ± 1.8[e]	49.7 ± 0.8	-31.9 ± 1.1	86.7 ± 1.2[h]	45.6 ± 0.0	-34.3 ± 0.2	92.1 ± 1.2
12	22.5	143.2 ± 2.4	-18.8 ± 1.0	37.2 ± 0.2[f]	91.2 ± 0.5	-30.7 ± 0.2	79.7 ± 1.2[h]	43.0 ± 0.6	-34.4 ± 0.1	92.8 ± 0.1
13	25	192.7 ± 2.7	-16.8 ± 0.0	34.5 ± 1.2[f]	95.2 ± 0.3	-30.3 ± 1.1	77.1 ± 0.6[h]	39.4 ± 0.4	-34.3 ± 0.1	93.4 ± 0.2

Table 3.6 Key physical properties of soluble whey protein aggregates formed on thermal treatment of 8% (w/w) WPI solutions (pH 6.0, 6.4 and 6.9) at temperature = **85 °C** for different times. *(Letters a,b,c,d and e means statistically different* ($P \leq 0.05$)*.*

		pH 6.0			pH 6.4			pH 6.9		
SN.	**Heating time (min)**	**Z-avg dia (nm)**	**ζ-potential (mV)**	**Soluble WPI aggregates (%)**	**Z-avg dia (nm)**	**ζ-potential (mV)**	**Soluble WPI aggregates (%)**	**Z-avg dia (nm)**	**ζ-potential (mV)**	**Soluble WPI aggregates (%)**
1	native	**12.3 ± 1.8**	**- 39.3**	**N/A**	9.8 ± 1.3	**- 41.2**	**N/A**	6.8 ± 1.5	**- 42.3**	**N/A**
2	1	87.0 ± 3.8	-28.3 ± 0.0	87.2 ± 0.0^a	43.5 ± 1.8	-29.2 ± 0.2	88.1 ± 0.2^d	40.5 ± 1.4	-35.7 ± 2.9	97.1 ± 0.2
3	2.5	97.4 ± 1.4	-29.3 ± 1.3	86.9 ± 0.2^a	45.6 ± 1.0	-28.8 ± 0.1	86.2 ± 0.1^d	42.1 ± 1.1	-33.9 ± 0.0	97.2 ± 0.1
4	4	100.6 ± 1.0	-28.1 ± 0.7	87.1 ± 0.1^a	49.8 ± 1.6	-29.4 ± 0.3	89.3 ± 0.1^d	40.3 ± 1.1	-34.0 ± 0.8	97.6 ± 0.2
5	5	108.2 ± 0.3	-28.3 ± 0.3	87.3 ± 0.1^a	61.0 ± 5.7	-29.4 ± 0.3	88.2 ± 0.2^d	41.4 ± 0.3	-33.9 ± 0.2	97.1 ± 0.3
6	7.5	108.5 ± 0.3	-28.3 ± 0.3	86.7 ± 0.2^a	56.3 ± 4.2	-28.9 ± 0.1	87.8 ± 0.3^d	43.2 ± 0.2	-34.0 ± 0.1	97.0 ± 0.1
7	10	112.3 ± 2.5	-29.6 ± 1.8	85.1 ± 0.2^a	57.1 ± 1.3	-29.1 ± 0.5	87.2 ± 0.4^d	48.9 ± 0.9	-34.5 ± 0.2	96.8 ± 0.4
8	12.5	115.4 ± 0.1	-28.2 ± 1.1	83.2 ± 0.2^b	60.4 ± 3.1	-28.4 ± 0.3	85.5 ± 0.1^e	45.8 ± 1.7	-34.6 ± 0.7	96.4 ± 0.4
9	15	118.2 ± 0.5	-29.3 ± 2.9	82.3 ± 0.1^b	58.2 ± 4.6	-29.5 ± 0.5	85.8 ± 0.1^e	59.1 ± 0.4	-34.0 ± 0.8	96.2 ± 0.1
10	17.5	120.7 ± 0.1	-30.0 ± 0.5	82.3 ± 0.0^b	59.9 ± 1.0	-29.0 ± 0.2	84.6 ± 0.1^e	59.7 ± 0.9	-34.3 ± 0.0	95.8 ± 0.1
11	20	123.0 ± 1.4	-27.0 ± 0.1	81.5 ± 0.2^b	59.9 ± 2.1	-29.2 ± 0.4	84.2 ± 0.0^e	63.3 ± 1.9	-35.4 ± 0.5	95.6 ± 0.6
12	22.5	125.2 ± 1.1	-25.6 ± 0.4	79.6 ± 0.0^c	59.8 ± 1.4	-29.3 ± 0.9	83.9 ± 0.0^e	62.1 ± 0.2	-35.0 ± 1.1	95.5 ± 0.3
13	25	126.9 ± 1.9	-26.1 ± 0.1	78.5 ± 0.1^c	57.4 ± 4.6	-28.6 ± 0.1	83.5 ± 0.2^e	53.0 ± 1.1	-35.3 ± 0.0	95.1 ± 0.1

At 68°C

The proportion of SWPA was influenced ($P \leq 0.05$) by pH of the solution and heat treatment time, especially at pH 6.0 and 6.4 compared to pH 6.9 (Table 3.4 and Fig 3.1A). However, the difference was small and was not highly noticeable. The results showed that the amount of whey protein (aggregates and native protein) remained as soluble in the supernatant after heat treatment at 68^{o}C after 60 min was 89.1, 90.0, and 94.0 % at pH 6.0, 6.4 and 6.9 respectively. At the heating temperature of 68°C, β-lg does not undergo complete denaturation even after extended duration of heating (> 48 hrs) as indicated in the earlier studies (Hoffmann & van Mil, 1999; Kazmierski & Corredig, 2003; Verheul et al., 1998). Formation of whey protein aggregates is influenced by the presence of α-lactalbumin (α-la). While α-la does not form aggregates on its own due to lack of free thiol group (Wijayanti et al., 2019), β-lg may undergo partial denaturation in the presence of α-la (Calvo et al., 1993; Quevedo et al., 2020). In a study at a similar concentration (10% w/v) of whey proteins carried out by Kazmierski and Corredig (2003) on heating at 68°C and pH 7, more than 60% β-lg remained in its native state even after 60 min of heating. A similar set of results has also been shown for a comparable concentration (5% w/v) of β-lg by (Hoffmann & van Mil, 1999) when the solution was heat treated at 65°C at different time intervals. The solution was adjusted to a pH of 6.0 to 8.0.

Although barely significant, solubility of whey protein aggregates at the end of heating (time = 60 min) decreased from 94% (pH = 6.9) to 90.1 (pH = 6.4) to 89.1% (pH = 6.0) **(Figure 3.1A)**. The decrease in the solubility was due to the formation of larger aggregates on account of reduction in repulsive forces between aggregates. In a study reported by Hoffmann et al. (1996), a higher amount of precipitates was observed when heating β-lg (5% w/v) in solution (pH = 6.2) at 68.5^{o}C for 5 hrs compared to pH = 6.4, 6.8 and 7.0. Higher protein concentration also leads to much more interaction among protein molecules and formation of larger aggregates as indicated by studies at temperatures close to 68 °C (Roefs & De Kruif, 1994). Much lower drop in solubility of whey protein aggregates at pH = 6.0 when heated at 68°C as compared with 75°C (Figure 3.1B) and 85°C (Fig 3.1C) could be attributed to much higher refolding of protein aggregates on cooling (Tolkach & Kulozik, 2007) which leads to higher values of solubility of aggregates. Although it is not sure, non-

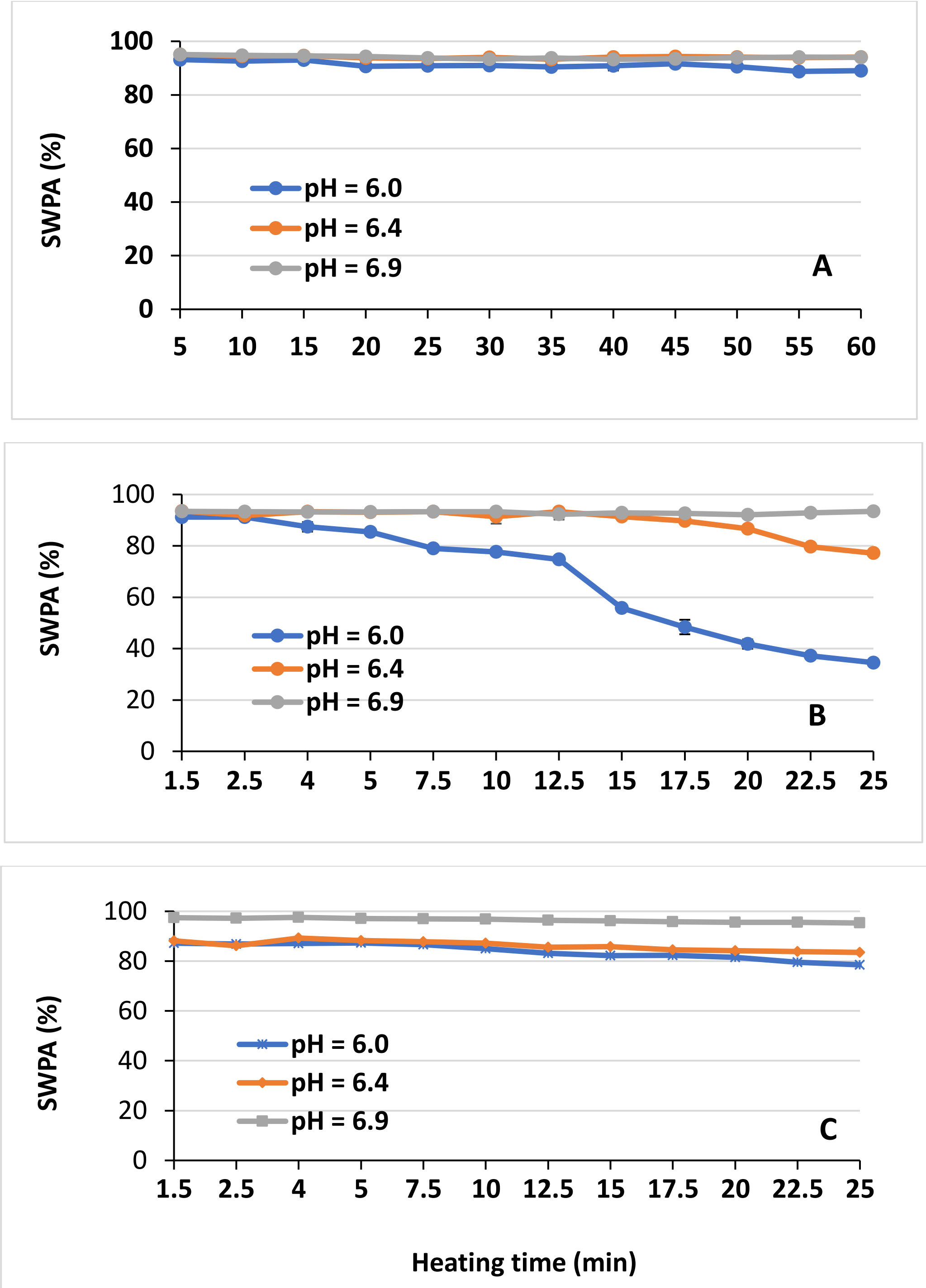


Figure 3.1 Effects of heating time and pH of WPI solution on the amount (yield) of soluble whey protein aggregates (SWPA) formed at (A) **68°C,** (B) **75°C** and (C) **85°C**.

covalent interactions which could lead to the formation of much bigger aggregates is largely absent at this heating temperature (< 75°C) (de la Fuente et al., 2002; Nicolai et al., 2011). However, some studies do speculate the presence of non-covalent interactions due to larger mass of aggregates formed at pH = 6.0 (Hoffmann & van Mil, 1997).

At 75°C

The proportion of soluble whey protein aggregates formed on heating at 75°C was significantly affected by pH prior to heating ($P \leq 0.05$) as depicted in **Figure 3.1B**. The duration of heating was also statistically significant ($P \leq 0.05$) for WPI solution maintained at pH = 6.0. The duration of heating did not affect the solubility at pH = 6.9 and 6.4. At heating temperature of 75°C, which is above the denaturation temperature of β-lg and whey protein isolate (72°C), it has been shown by several studies that almost 80% of protein is denatured within a few minutes (≈ 5 to 10 minutes) at near neutral conditions (Kazmierski & Corredig, 2003; McSwiney et al., 1994; Monahan et al., 1996). At pH = 6.9, much quicker formation of aggregates at approx. 2.5 minutes observed in this study compared to slightly longer time recorded in the earlier studies could be attributed to higher protein concentration which allows more sites for intermolecular and intramolecular interaction between free thiol and disulphide bridges. Formation of much larger aggregates or insoluble precipitates was not observed at pH = 6.9.

For pH = 6.4, on heating at 75°C for 15 and 25 min, the solubility decreased gradually from 91.4% to 77.1%, respectively. The proportion of soluble whey protein aggregates was observed to be reduced even further as the pH of solution was reduced to 6.0 prior to heating. The proportion of soluble whey proteins was reduced drastically to 34.5% on heating at 75 °C for 25 min. As indicated in **Table 3.5 and Figure 3.1 B**, the solubility of aggregates reduced sharply with heating time for pH = 6.0. A reduction in the amount of soluble aggregates at pH = 6.4 could be on account reduction in repulsive forces and also an increase in participation of α–la (Matsudomi et al., 1992; Verheul et al., 1998). It has been noted earlier by Verheul et al. (1998) that the rate of denaturation of β–lg was reduced on heating at 75°C as the pH of solution before heating was reduced from 6.0. The study also noted an increase in the mass of aggregates as pH was reduced to pH = 6. A sizable amount of precipitation at pH =6.0 due to insoluble aggregates on heating at 75°C as compared to 68°C can also be ascribed to much larger role of non-covalent interactions at this temperature (Iametti et al., 1996). In a study by

(Dalgleish et al., 1997), it was noted that in the presence of α–la, β–lg forms much larger aggregates. It also noted that if the proportion of denatured α–la is relatively higher than denatured β–lg, the solution formed gels at pH = 6.0 compared to pH = 7.0. The heating temperature used in this study was 75°C and used varying ratio of α–la/ β–lg. Possibly, a combination of lower rate of denaturation and presence of denatured α – la could have led to much lower values of solubility unlike those observed for 68°C and 85°C and at pH = 6.0. The earlier studies have noted that mechanism of aggregate formation of mixtures of α–la and β–lg on heating at 75°C are different from those for standalone proteins as well as other heating temperatures (Havea et al., 2001; Havea et al., 1998; Matsudomi et al., 1992; Verheul et al., 1998) . However, the underlying reasons remain unknown.

The reduction in repulsive forces with reduction in pH could also contribute to formation of larger aggregates. Besides, it was shown that higher WPI concentration facilitates the formation of larger aggregates as repulsive forces was reduced with reduction in pH (Xiong et al., 1993). The formation of larger aggregates leading to much rigid and turbid WPI gels has also been reported by Lorenzen and Schrader (2006) at pH = 6 on heating WPI solution of 11.25% (w/w) at 75 °C for 45 min. The gels obtained at pH = 7 were shown to be translucent. Large aggregates observed on heating for a duration greater than 10 min in the present study as the pH was lowered from 6.9 to 6.0 can be compared with coagulum observed during formation of turbid gels. As the concentration of proteins used in this study was 7.5% w/w (measured based on WPI concentration of 8% w/w) which was less than the critical gel concentration of 10% w/w at pH = 6 (Ako et al., 2009; Boland, 2011; Schokker et al., 1999), a large network of aggregates leading to gelation could not be formed.

At 85°C

In case of heat treatment at 85°C, only the pH of solution was found to have a significant effect ($P \leq 0.05$) (**Table 3.6 and Figure 3.1C**) on the solubility of whey protein aggregates. At neutral pH, both WPI and β-lg are in completely denatured state when heated at heating temperatures ≥ 85°C as noted in the previous studies reported by Schmitt et al. (2007) and Croguennec et al. (2004) In this study, at the end of heat treatment for 25 min, the proportion of soluble aggregates decreased from approx. 95% at pH = 6.9 to 83.5 % at pH = 6.4 to 78.5% at pH = 6.0 as shown in **Figure 3.1C**. The downward trend in the solubility is similar in comparison with the trend observed at 68°C and 75°C. The reduction in the solubility at pH =

6.0 indicates the formation of larger insoluble aggregates compared to those formed at pH = 6.4 and 6.9. The proportion of larger insoluble aggregates with reduction in pH observed in this study compares well with the values observed at smaller concentration of WPI (1% w/w) reported by Schmitt et al. (2007) and larger concentration of β-lg (5% w/w) by Zúñiga et al. (2010). Both studies were conducted with a similar pH range of 6-7 prior to heating at 85°C and the proportion of larger insoluble aggregates was detected by use of SDS-PAGE. The proportions of insoluble aggregates noted by Zúñiga et al. (2010) were 15% (pH = 6.9), 17% (pH = 6.4) and 22% (pH = 6.0) on heating at 80°C for 15 min. Formation of larger aggregates has been attributed to the presence of non-covalent interactions in these studies. In comparison with the solubility of protein aggregates at pH = 6.0 on heating at 75°C, higher values of solubility at pH = 6.0 on heating at 85°C can be attributed to much faster rate of denaturation at heating temperatures > 80 °C (Law & Leaver, 2000; Tolkach & Kulozik, 2007; Zúñiga et al., 2010). In such a scenario, aggregation becomes a rate limiting step.

3.4.2 Particle size of soluble whey protein aggregates

In this study, the supernatants obtained after centrifuging the heated WPI solutions are defined as a stable, colloidal dispersion of water-soluble whey protein aggregates. The particle size of aggregates was measured based on dynamic light scattering technique and expressed as Z-average diameter (ZD) which is the intensity weighted mean hydrodynamic size. The particle size distribution of aggregates was also expressed as a frequency distribution of particles (volume frequency v/s D_{43}).

Unheated or native WPI particles had a ZD of 6.8, 9.8 and 12.8 nm for pH = 6.9, 6.4 and 6.0, respectively (**Tables 3.4-3.6**). The size of unheated WPI particles confirms well with the previous studies reported by some researchers (Dalgleish & Leaver, 1991; Mahmoudi et al., 2007; Zúñiga et al., 2010). The heating temperature did not have a significant effect on the size of aggregates when the value of pH was held at pH = 6.9. The ZD values of whey protein aggregates at the end of heating cycle at pH = 6.9 was 46.2 nm, 39.4 nm and 53 nm on heating at 68°C, 75°C and 85°C, respectively (**Figure 3.2 & Tables 3.4-3.5**). These results compare well with several previous studies at varying concentrations of whey proteins (Britten & Giroux, 2001; Jung et al., 2008; Kazmierski & Corredig, 2003; Roefs & De Kruif, 1994; Schmitt et al., 2007; Zúñiga et al., 2010).

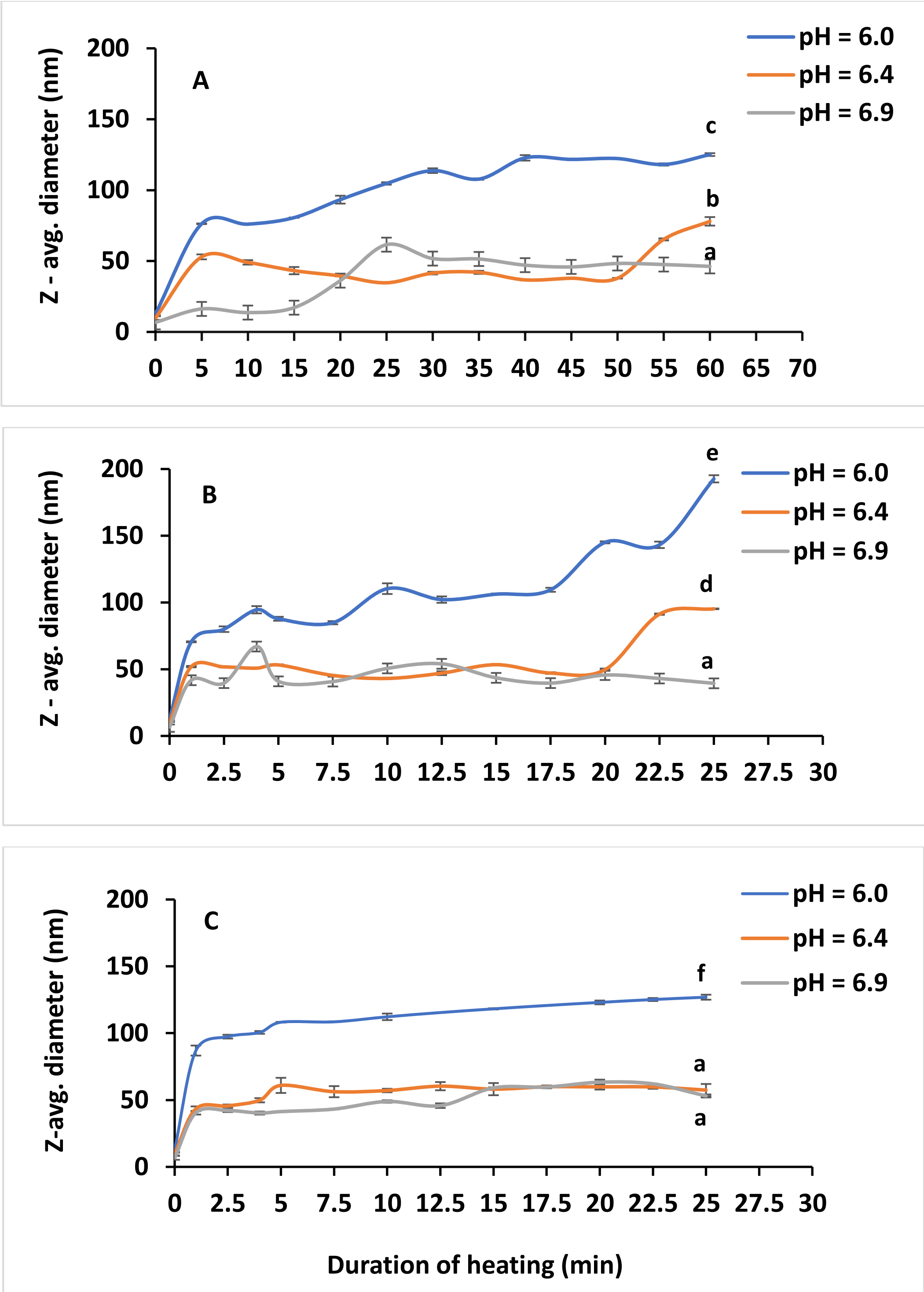


Figure 3.2 Effect of heating time and pH of WPI solution on particle size of whey protein aggregates formed at (A) **68°C,** (B) **75°C** and (C) **85°C**. Heating time = 0 depicts WPI in native form. *(Letters a,b,c,d,e and f means statistically different* (P ≤ 0.05).

The repulsive forces were sufficiently large at this pH value (pH 6.9) which was sufficiently away from the isoelectric point of whey proteins (≈ 5.1) to prevent the formation of much larger aggregates.

For all heating temperatures, the pH of WPI solutions prior to heating had a significant effect ($P \leq 0.05$) on the ZD values of aggregates. The reduction in pH led to reduction in repulsive forces which enhanced further aggregation and increase in particle size for all three different heat-treatment temperatures **(Figure 3.2)**.

At 68°C, the formation of aggregates took much longer, and the presence of whey protein aggregates was noted only after a much longer duration of 20 min heating time (**Figure 3.1A and Figure 3.3A**). This was in agreement with some previous studies at these condition (Britten & Giroux, 2001; Hoffmann & van Mil, 1999; Kazmierski & Corredig, 2003; Roefs & De Kruif, 1994) which noted a slightly longer duration of 40 min for the formation of such aggregates. An earlier study reported by Alting et al. (2003) on heating the solution of WPI (pH =7) at 68.5°C noted that the time required to achieve 95% protein aggregation was reduced from 24 h to 4 h as the WPI concentration was increased from 3% (w/w) to 7.5% (w/w). An increased WPI concentration appears to have accelerated the formation of intermolecular disulphide bonds which led to early presence of aggregates and reduced the time required for aggregate formation to 20 min. In two independent studies reported by Iametti et al. (1996) and Tolkach and Kulozik (2007) at a much smaller concentration of β-lg, it was found that a large portion of denatured proteins refolded back to the native state upon cooling after heating at 70°C. The aggregates at 68°C which is lower than denaturation temperature of β-lg is on account of involvement of α – la in the formation of aggregates. This has been noted at similar heating temperature (65°C) by Kazmierski and Corredig (2003) with WPI at 10% w/v. The involvement of α–la can be assumed at all pH values. As the pH was reduced to 6.4 and further to 6.0, the size of aggregates became bigger as the heating time increased as indicated in **Table 3.4**. The ZD value of aggregates was gradually increased with heating time to 78.0 nm for pH = 6.4 and 125 nm for pH = 6.0 as depicted in **Figure 3.1A**. The presence of larger protein aggregates with increasing the time of heat treatment was also evident from the size distribution of particles for pH 6.4 **(Figure 3.3B)** and pH 6.0 **(Figure 3.3C).** For β-lg alone, it has been found in earlier studies that as pH is reduced from 8.0 to 6.0, the rate of denaturation slows down even further (Hoffmann & van Mil, 1999; Tolkach & Kulozik, 2007). In fact, volume mean diameter (obtained from size distribution) has also been noted at 80°C in a study on reported on β-lg (5% w/v) alone by Zúñiga et al. (2010) and at 90°C by Ryan et al. (2012).

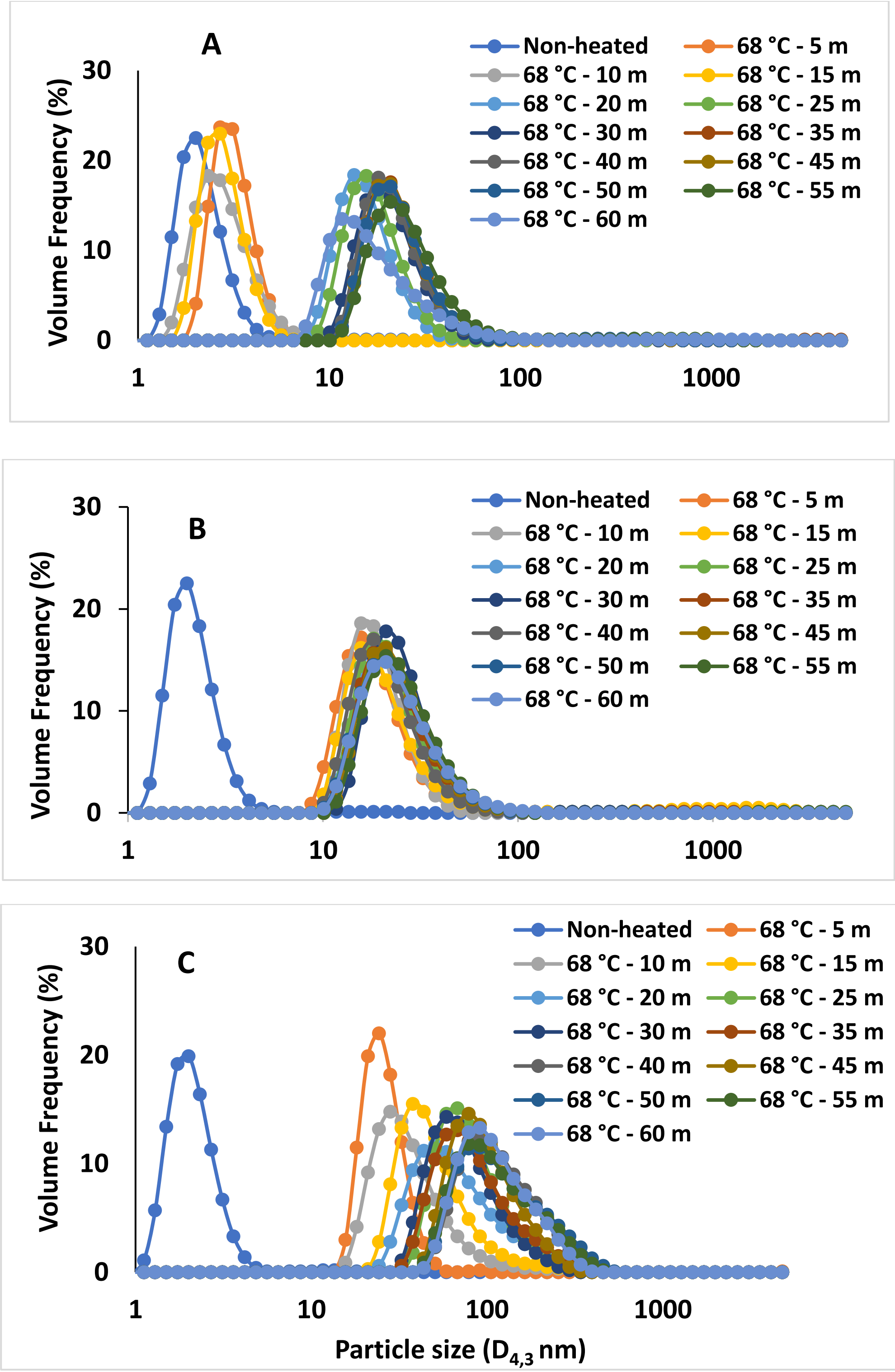


Figure 3.3 Particle size distribution of whey protein aggregates formed after thermal treatment of 8% (w/w) WPI solution at **68°C** for different heating times. (A) pH = 6.9, (B) pH = 6.4 and (C) pH = 6.0

Hoffmann and van Mil (1999) observed at heating temperature of 65°C, a large amount of protein remained in native form. There is no data available for reaction rates of α–la at this heating temperature and varying pH values. In an earlier study by Hoffmann and van Mil

(1997) on heating β-lg at 65°C at a concentration of 1- 5% w/v noted that while the number of aggregates formed at lower pH (<6.4) was smaller, the mass of aggregates calculated based on SDS-PAGE was much larger. The aggregates formed at pH = 6.4 to 8.0 are largely due to covalent bonds. However, very large aggregates formed at pH = 6.0 consists of both covalent and non-covalent bonds. In a study by Verheul et al. (1998), the heat treatment of solution with a lower concentration of β-lg at 65°C and 68.5°C was shown to have a lower denaturation rate at pH = 6.0 but it was increased with an increase in pH. Each of these studies noted that β-lg largely remained in undenatured form at pH = 6.0. Unlike heating β-lg alone, the size of WPI aggregates is influenced by α–la. The larger aggregates formed at pH = 6.0 could be largely due to reduction in repulsive forces as it remains unknown if presence of large amount of α–la compared to β-lg could influence formation of aggregates at heating temperature of 68°C. It has been shown to have an impact of formation of aggregates at higher heating temperature of 75°C and pH = 6.0 (Dalgleish et al., 1997)

At isothermal heating conditions, the duration of heating time did not appear to affect the particle size in a uniform manner. The effect of duration of heating on the size of aggregates varied with different levels of pH prior to heating. The duration of heat treatment did not seem to have a significant effect for the temperature of 75°C and 85°C when the pH of solution was at pH = 6.9 and the presence of aggregates could be detected merely after 2.5 min of heat treatment based on the size distribution of aggregates as shown in **Figure 3.4A and Figure 3.5A,** respectively. The presence of aggregates at much shorter interval compared to 68°C is because denaturation occurs very fast at these conditions and the rate of aggregation is the rate limiting step at this pH (Croguennec et al., 2004; Mounsey & O'Kennedy, 2007). This has also been confirmed by Iametti et al. (1996) with much shorter half-life (< 110 sec) of β-lg dimers at temperature > 75°C. While there was hardly any significant change in the ZD values of aggregates as the heating proceeded, a broader distribution of aggregate sizes was visible from **Figure 3.4 B**. This difference between ZD

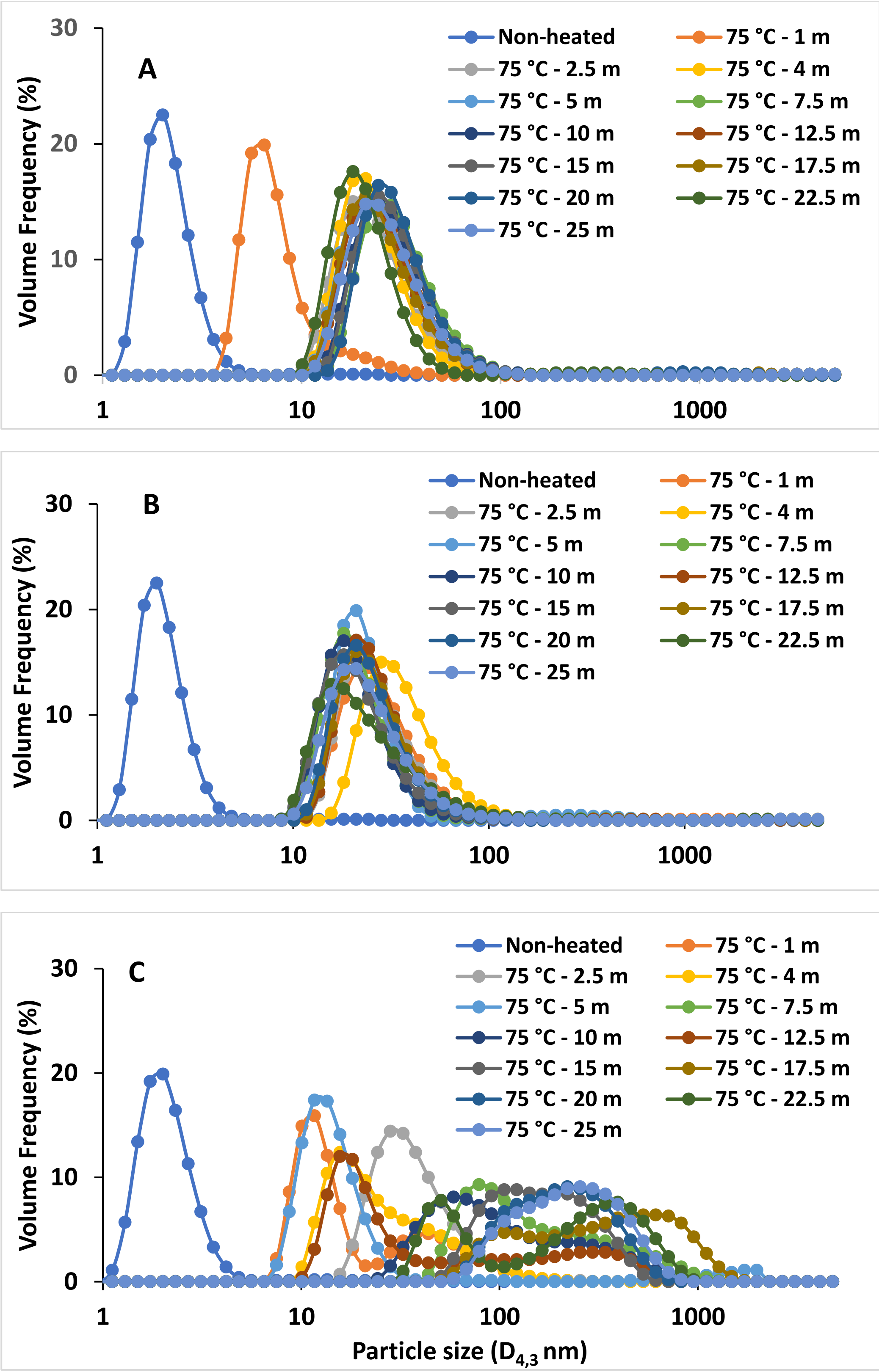


Figure 3.4 Particle size distribution of whey protein aggregates formed on thermal treatment of 8% (w/w) WPI solution at **75°C** for different heating times. (A) pH = 6.9, (B) pH = 6.4 and (C) pH = 6.0

A gradual increase in the particle size of soluble aggregates as heating proceeded at pH = 6.4 and 6.0 for heating temperature of 75°C **(Figure 3.4 B and C)**. For heating at 75°C, the particle size of aggregates obtained at the end of heating (25 min) at pH = 6.4 was 95.2. The ZD of soluble aggregates formed at pH = 6.4 increased from 51.9 nm at t = 2 min to 95.2 nm at t = 25 min (**Figure 3.1B and Table 3.5**). The particle size distribution depicted in **Figure 3.4B** shows a gradual increase in particle size as heating proceeded. This was higher than that obtained at pH = 6.9 (ZD = 39.4) **(Table 3.5)**. At near neutral conditions, it has been observed in the earlier studies that smaller aggregates are formed on account intermolecular disulphide bonds between dominant whey protein components (β – lg and α – la) (Dalgleish et al., 1997; Havea et al., 2001). The increase in size of aggregates at pH = 6.4 is on account of reduction in repulsive forces. The aggregates formed at this pH are also linked by intermolecular bonds (Verheul et al., 1998). As the pH is reduced to 6.0 and the solution is heated at 75°C, a combination of two phenomenon could lead to formation of much larger aggregates. The rate of denaturation of β-lg at the heating temperature of 75 °C reduced as pH is reduced to 6.0 (Verheul et al., 1998). While one would assume that with slower denaturation rate, there would be large presence of native β-lg, the presence of much larger denatured α – la at pH = 6.0 compared to denatured β-lg leads to formation of very large aggregates on account of non-covalent bonds (Dalgleish et al., 1997). This led to a gradual increase in the size of aggregates (ZD = 71 nm at t = 1 min to ZD = 193 nm at t = 25 min) **(Table 3.5)** through both covalent and non-covalent interactions. The presence of much larger aggregates with the duration of heating is clearly visible in **Figure 3.4C**. A much broader distribution of particle sizes when the duration of heat treatment was more than 10 min indicates the presence of larger clusters of aggregates. A gradual creep towards bigger particle size was also evident from the particle distribution shown in **Figure 3.4C.**

The ZD values of aggregates were 57.4 nm and 126.9 nm on heating at 85°C when the pH of WPI solution was at pH = 6.4 and 6.0, respectively **(Figure 3.5B and C)**. The particle size distribution obtained at these heat treatment conditions confirms that the duration of heating did not have a pronounced effect the particle size of whey protein aggregates at a constant level of pH. The formation of larger aggregates when the pH was decreased could be aided by a reduction in repulsive forces. These results were in agreement with the previous studies on WPI (Schmitt et al., 2007) at 1% w/w and on β-lg (Zúñiga et al., 2010) at 5% w/w.

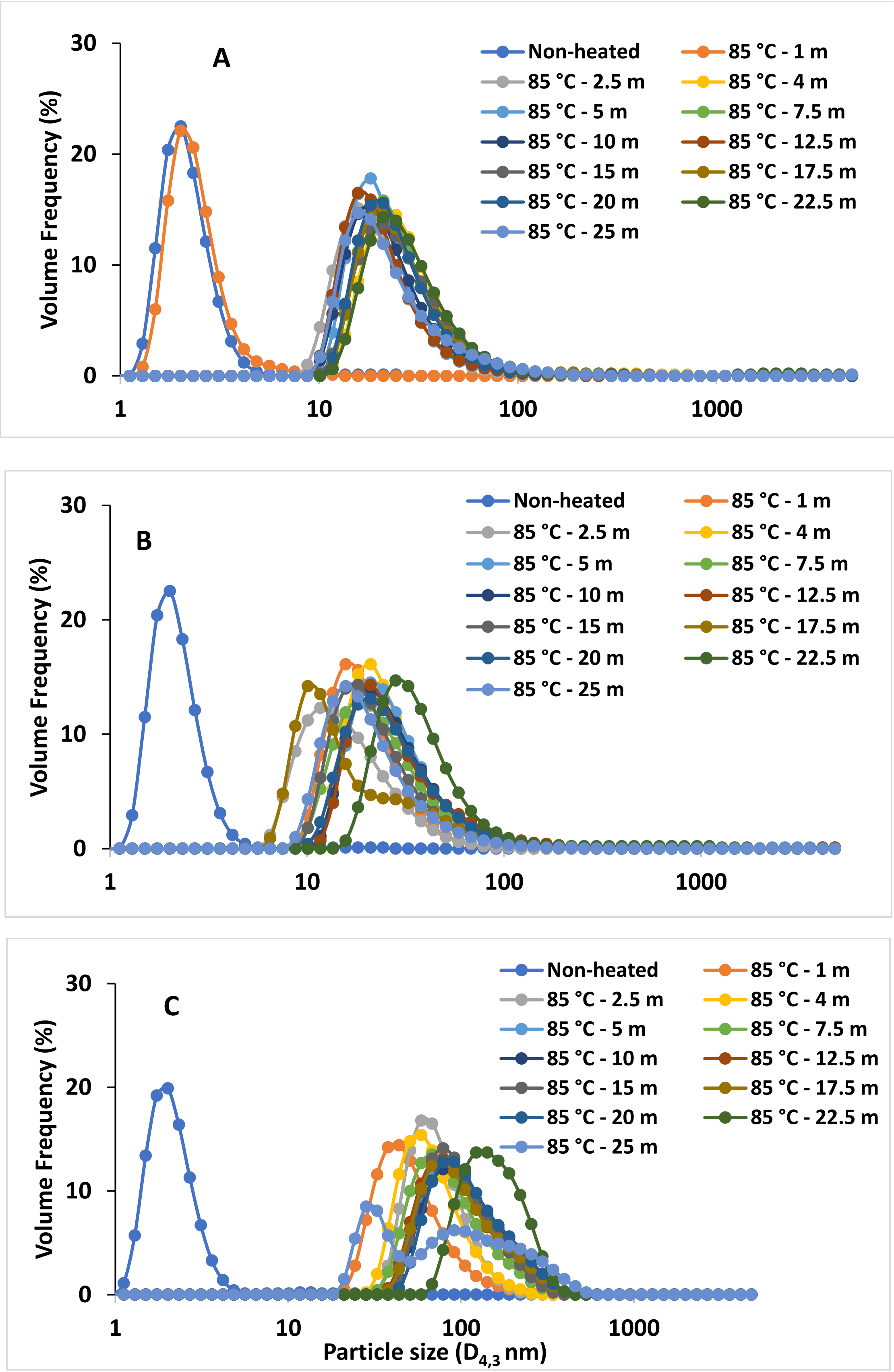


Figure 3.5 Particle size distribution of whey protein aggregates formed on thermal treatment of 8% (w/w) WPI solution at **85°C** for different heating times. (A) pH = 6.9, (B) pH = 6.4 and (C) pH = 6.0

Although it is not sure as the experimental conditions are not same, it seems protein concentration may not have a significant role in the formation of aggregates at an elevated temperature of 85°C as the results of particle size obtained in this study confirm well with the previous studies conducted at much lower protein concentrations. The rate of denaturation has been found to increase with reduction in pH at this temperature by Law and Leaver (2000) on skim milk and by Zúñiga et al. (2010) for β-lg which make aggregation a rate limiting step.

3.4.3 ζ-potential

Zeta (ζ)-potential values were obtained based on the electrophoretic mobility of soluble whey protein aggregates in the dispersion. The term "soluble whey protein aggregates" was defined in this study as "colloidal" particles retained in the supernatant of heat-treated WPI solutions after centrifugation. While analysing the ζ-potential values for aggregates in the supernatant solutions, it is important to note that the measurement of electrophoretic potential assumes a spherical structure of particles (Analytical, 2013). Duration of heating did not appear to have a significant effect for the heat treatment of 68°C when the pH of WPI solution was 6.9 but there was a gradual decline in the values of ζ-potential from -34.9 mV to -27 mV as heating proceeded at pH = 6.4 as indicated in **Table 3.4 and Figure 3.6**. A higher value at the initial stage of heating could be due to the presence of native proteins in the dispersion. Duration of heating did not have a significant impact on samples at pH = 6.0 after heating more than 25 min and the values remained relatively uniform afterwards.

As indicated in **Table 3.5 and Fig 3.7**, for heating at 75°C, ζ-potential values remained uniform for pH = 6.9 with no significant changes from -34.5 mV to -34.3 mV and for pH = 6.4 with marginal change in values from -34.6 mV to -30.6 mV with increasing duration of heat treatment. However, duration of heating had a significant impact ($P \leq 0.05$) on the values of zeta potential at pH = 6.0. A significant reduction in ζ-potential from -28.7 mV at t = 1 min to -16.8 mV at t = 25 min could be due to the presence of much larger aggregates in the dispersion. For the samples heat-treated at 85^{o}C, as indicated in **Table 3.6 and Figure 3.8**, the duration of heating or pH did not have a significant effect on the ζ-potential values. The values obtained for soluble whey protein aggregates formed at different levels of pH indicate a substantial level of repulsive force acting on water soluble protein aggregates which would render a long-term stability of dispersion against separation and precipitation.

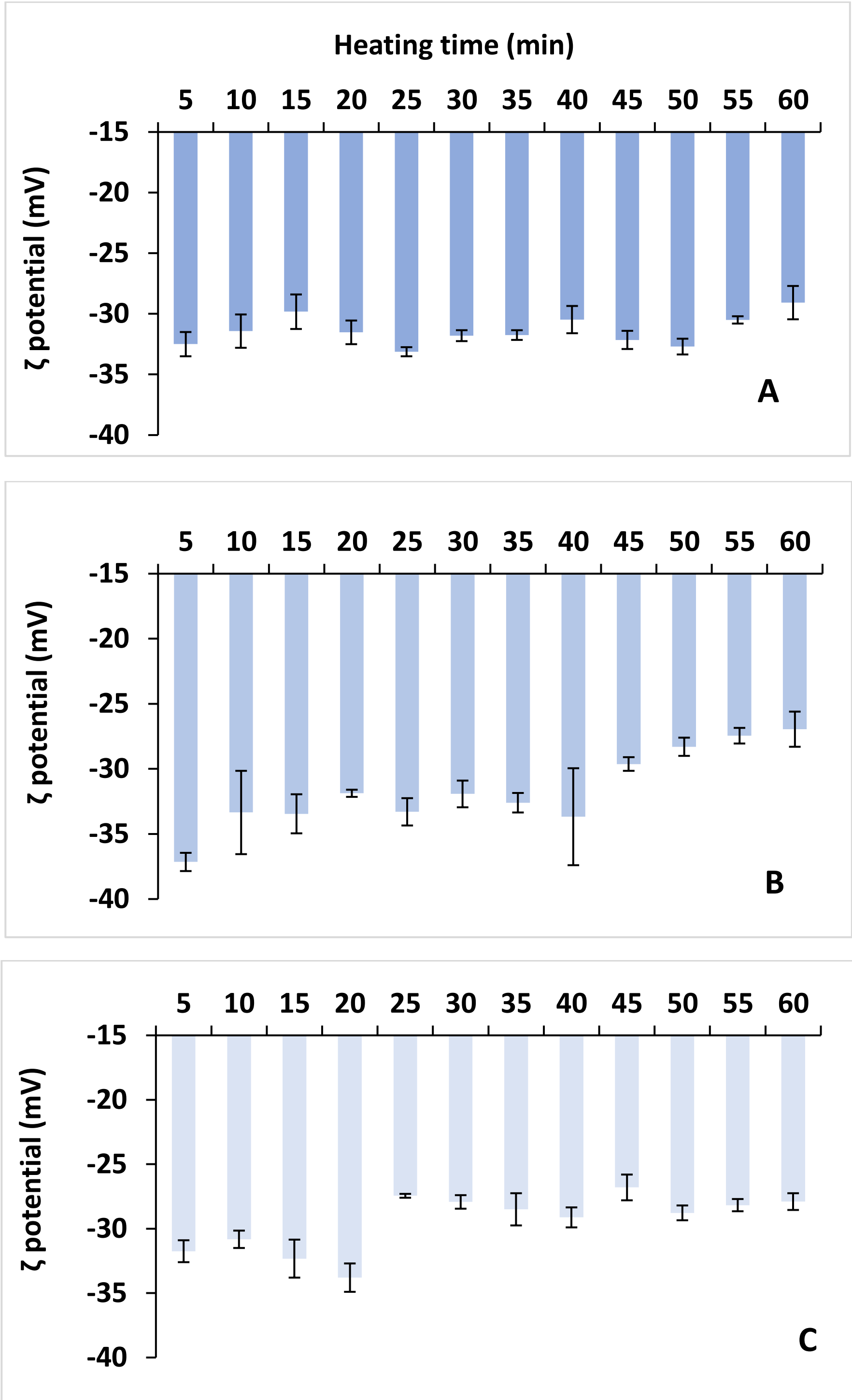


Figure 3.6 ζ-potential of whey protein aggregates formed on thermal treatment of 8% (w/w) WPI solution at **68°C** for different heating times. (A) pH = 6.9, (B) pH = 6.4 and (C) pH = 6.0.

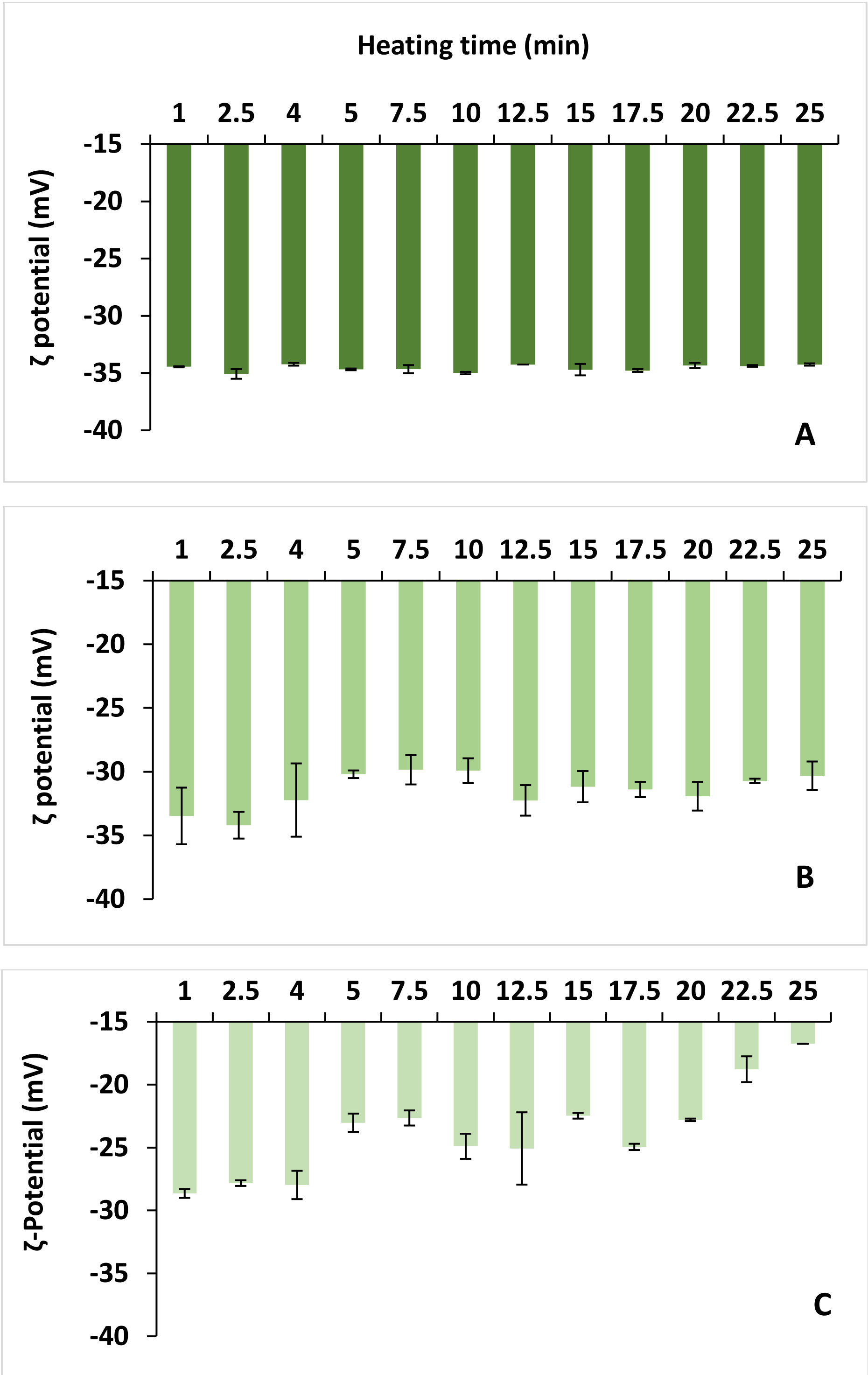


Figure 3.7 ζ-potential of whey protein aggregates formed on thermal treatment of 8% (w/w) WPI solution at **75°C** for different heating times. (A) pH = 6.9, (B) pH = 6.4 and (C) pH = 6.0.

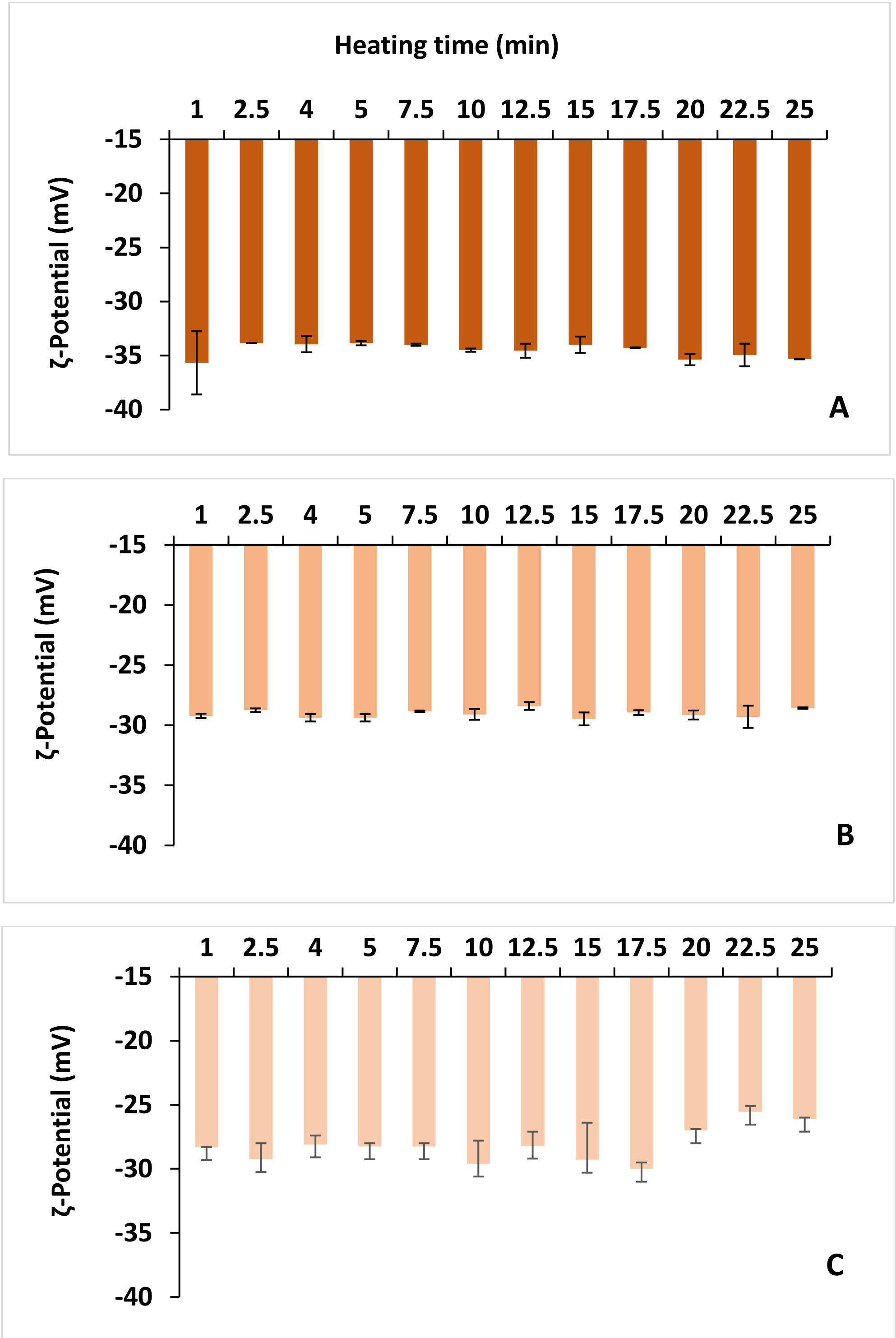


Figure 3.8 ζ-potential of whey protein aggregates formed on thermal treatment of 8% (w/w) WPI solution at **85°C** for different heating times. (A) pH = 6.9, (B) pH = 6.4 and (C) pH = 6.0

3.5 Conclusions

The characteristics of soluble whey protein aggregates indicated by the % solubility and particle size of soluble aggregates were found to be significantly affected by the pH of WPI solution prior to its heat treatment. The particle size of soluble whey protein aggregates obtained at the end of heating at 68, 75 and 85°C increased with the reduction of pH from 6.9 to 6.0 before the start of heating. Also, the yield or proportion of soluble whey protein aggregates (% solubility) obtained after heat treatment was not only influenced by pH and but also by heating temperature. It is evident from the study that effect of pH is not uniform across all heating temperatures. Although at a broader level, the study did observe a secular trend towards the formation of larger aggregates with reduction in pH from 6.9 to 6.0, the extent of insoluble precipitate was equally dependent on underlying reaction kinetics. It should be noted that the amount of larger insoluble aggregates observed at a particular heating temperature was influenced by the nature of rate limiting reaction (i.e. denaturation or aggregation). The yield of soluble whey protein aggregates decreased substantially to 34.5% as the heating proceeded at 75°C and at pH = 6.0. This clearly indicates an intense aggregation of unfolded whey proteins as denaturation occurred slowly at lower pH. A combination of slower denaturation rate of β–lg and extensive non-covalent interactions between β-lg and α–la led to the formation of a higher amount of insoluble aggregates at this process conditions. The yield of soluble whey protein aggregates (% solubility) was much higher (approx. 90%) on heating at 68°C and remained fairly consistent in spite of changes in pH. The experimental work was able to achieve that a stable dispersion of whey protein aggregates with a yield of more than 80% soluble aggregates could be achieved at 85°C and at the end of 25 min of heat treatment. The particle size of 53.0, 57.4 and 126.9 nm was obtained at pH = 6.9, 6.4 and 6.0, respectively, when heating at 85°C for 25 min. These particles could be considered as useful candidates for further investigation of their use as an emulsifier in O/W emulsions. A future study could also involve comparison between native WPI and soluble whey protein aggregates for their properties in forming and stabilising O/W emulsions. Future work should also focus on characterisation of soluble whey protein aggregates using microscopy to understand the microstructure of these particles to enable its wider use in real world food applications.

Chapter 4. Effect of particle size of water-soluble whey protein aggregates on the characteristics of O/W emulsions

4.1 Abstract

Soluble whey protein aggregates of varying particle size (40 to 140 nm) were prepared by thermal treatment of whey protein isolate (WPI) solution with a high WPI concentration of 8% w/w. O/W emulsions were prepared with constant oil content of 15% w/w to obtain oil droplets with a hydrodynamic diameter of < 500 nm. The O/W emulsions stabilised by soluble whey protein aggregates were compared for their physical characteristics like droplet size, ζ-potential and apparent viscosity with the O/W emulsion stabilised by non-heated WPI. Particle size of whey protein aggregates had a significant effect on physical characteristics of O/W emulsions. The O/W emulsions were analysed for storage stability, change in pH, change in ionic strength and thermal treatment. The presence of high concentration of non-adsorbed whey protein particles not only influenced the physical characteristics but also affected the thermal stability of emulsions. An equally significant impact was observed when changing of pH and ionic strength on all O/W emulsions. While O/W emulsions stabilised by non-heated WPI remained a Newtonian fluid, the ones stabilised by soluble whey protein aggregates displayed a shear thinning behaviour with apparent viscosity of emulsions increasing with the particle size of whey protein aggregates. The O/W emulsions stabilised by soluble whey protein aggregates with particle size of approx. 50 nm displayed better stability to thermal treatment compared to emulsions stabilised by non-heated WPI and soluble whey protein aggregates with larger particle size of 133 nm. The O/W emulsions stabilised by soluble whey protein aggregates formed a kind of emulsion gels (semi-solid) in the pH range of 4 to 5 as well $CaCl_2$ concentration $\geq$ 5 mM. The results of the study suggest that soluble whey protein aggregates with varying particle size could be used to modify the rheology of high protein and low-fat emulsions and emulsion gels without use of additional rheology modifiers.

4.2 Introduction

The consumer preference to a high protein and reduced fat diet can be either a fleeting trend or a much more lasting dietary choice. A growing body of research work has attributed high protein intake as an important tool to combat the menace of lifestyle ailments like obesity, hypertension, type 2 diabetes and post work-out fatigue. There is equally persuasive research to opt for low fat diet as a lifestyle choice (Borreani et al., 2017; Burd et al., 2012; Layman et al., 2009). As per the 21 Code of Federal Regulation (CFR) guidelines laid down by U.S. Food and Drug Administration (FDA), a product can be labelled as "high" protein if it contains more than 10 gm of protein per 250 gm of serving which corresponds to the protein content of > 4%. The product can also be labelled as "high" protein if the protein content is more than 20% of Daily Recommended Value (DRV) of protein (FDA, 2021; Mulcahy et al., 2018; Singh et al., 2020). There is a broad consensus among nutritionists and dietary experts about the nutritional superiority of whey proteins compared to other forms of plant and animal proteins (Ali et al., 2019; Lang et al., 1998; Norton et al., 2021; Pennings et al., 2011). Whey proteins have been incorporated into several food products in order to prepare "high" protein versions of popular products like jams, yogurts, puddings and biscuits (Ahmed et al., 2019; Karam et al., 2013; Mulders, 2017; Vidigal et al., 2012). There have been much fewer attempts at use of whey protein in protein rich fluid emulsions (Liang et al., 2013; Liang et al., 2016). Milk proteins like whey proteins which are globular proteins have been used often to stabilise O/W emulsions for their ability to rapidly adsorb at the interface (protein diffusion rate), reduce the interfacial tension and provide stability by forming a closely packed thick interfacial layer of 2-3 nm (Dalgleish & Leaver, 1991; Dickinson, 2010; Murray & Dickinson, 1996; Nylander et al., 1999). The interfacial layer formed by whey proteins has been modelled as a planar gel layer (Dickinson, 2001) and stabilises emulsions by a combination of electrostatic and steric stabilisation (Dickinson, 1992b, 1998; Dickinson & Matsumura, 1994; D.J. McClements, 2015).

Heat induced whey protein aggregates have been extensively studied in the context of cold-set gelation (Barbut & Foegeding, 1993; Bryant & McClements, 1998; Bryant & McClements, 2000; de la Fuente et al., 2002) on account of its nutritional superiority compared to polysaccharides (Doi, 1993; Ministry of Health, 2015). The characteristics of whey protein aggregates in terms of particle size, mass or molecular weight, free thiol groups, and protein concentration have been shown to have a decisive impact on the characteristics (hardness, storage modulus, elastic modulus, etc.) of cold set whey protein gels (Alting et al., 2003; Alting et al., 2000; Bryant & McClements, 2000; Hongsprabhas & Barbut, 1997). There is a growing body of work in form of journal papers (Famelart et al., 2015; Kew et al., 2020; Loveday et al., 2010; Nicolai & Durand, 2013; Zhang et al., 2016) and some patents (Limited, 1990; Proteins, 2012) which have claimed water soluble whey protein aggregates as a suitable candidate for use as an emulsifier and stabiliser as well as a "fat replacer". The particle size of these aggregates has often been noted as an important parameter to modify the rheology of interfacial layer as well as its surrounding bulk phase (Schmitt et al., 2007; Vardhanabhuti & Foegeding, 1999). Although a number of studies and patents have extolled the desired characteristics of soluble whey protein aggregates, the characteristics of emulsions stabilised by soluble whey protein aggregates have not been studied extensively (Gélébart et al., 2019; Knudsen et al., 2008; Zhou et al., 2020) Similarly there have been very few studies that have investigated high protein or protein rich emulsions stabilised by soluble whey protein aggregates (Boutin et al., 2007; Chevallier et al., 2016).

Soluble whey protein aggregates have been prepared with their particle size of 40 – 130 nm by heat treatment at temperatures greater than the denaturation temperature of whey protein at a negligible ionic strength and at a pH, sufficiently away from the isoelectric point of whey protein (Kazmierski & Corredig, 2003; Roefs & De Kruif, 1994; Schmitt et al., 2007; Zúñiga et al., 2010). Soluble whey protein aggregates were also prepared in a similar particle range as depicted in results obtained in Chapter 3. Native whey proteins with β-lg as the most dominant protein have a typical particle size of 3 – 7 nm (Bauer et al., 2000; Dalgleish & Leaver, 1991; deWit & Klarenbeek, 1984). Several important properties of soluble whey protein aggregates, such as surface hydrophobicity, intrinsic viscosity, surface viscosity and emulsifying index, are not only influenced by size of the aggregates (Maticorena et al., 2018; Moro et al., 2013; Schmitt et al., 2007) but would also be extremely crucial in its successful use as an emulsifier or a fat replacer. A peculiar spherical structure termed "microgel" has been observed to form on account of thermal denaturation and aggregation of whey protein in solution at pH $\approx$ 5.8 (Donato et al., 2009) or at neutral pH in the presence of calcium ions (Phan-Xuan et al., 2013). A number of studies have contributed to improving the stability of emulsions against coalescence and flocculation by stabilising the emulsions with whey protein microgels resulting in the formation of Pickering emulsions (Destribats et al., 2014; Dickinson, 2012b, 2015; Sarkar et al., 2016). However, soluble whey protein aggregates which are typically curved or fibrillar in structure and much more soluble in water (Mehalebi et al., 2008; Nicolai et al., 2011) may not be suitable candidates for Pickering emulsions (Binks & Horozov, 2006; de Folter et al., 2012; Dickinson, 2012b). A recent study by Dybowska and Krupa-Kozak (2020) on emulsions formed by soluble whey protein aggregates has alluded to the formation of Pickering emulsion.

A few recent studies on O/W emulsions (Moro et al., 2013; Sobhaninia et al., 2016) and foams (Dombrowski et al., 2016; Rullier et al., 2009; Rullier et al., 2008) have found that whey protein aggregates with smaller particle size quickly diffuse through the bulk phase and efficiently cover the oil droplets. The slower rate of diffusion for larger aggregates is ascribed to much higher values of surface viscosity of dispersions (Garstecki et al., 2006; Nicolai et al., 2011; Schmitt et al., 2007) due to an increase in particle size (Maticorena et al., 2018). Almost every study in recent years related to emulsions stabilised by whey protein aggregates has noted shear thinning or non-Newtonian fluid characteristics (Boutin et al., 2007; Eissa et al., 2014; Genovese, 2012; Knudsen et al., 2008; Moakes et al., 2015). A number of factors like changes in pH, temperature, ionic strength, duration of storage, concentration of emulsifiers and etc have shown to destabilise O/W emulsions by creaming, gravitational sedimentation, flocculation and coalescence (Delahaije et al., 2015; Dickinson, 2010; Tadros, 2019; Tcholakova et al., 2006). In the context of high protein or protein rich emulsions, the presence of non-adsorbed protein in continuous phase has been shown to have a crucial effect on the stability of emulsions (Euston et al., 2000; Hunt & Dalgleish, 1995; Kulmyrzaev et al., 2000; Ye & Taylor, 2009). On thermal treatment (> 90^{o}C), the non-adsorbed proteins in the continuous phase form whey protein aggregates and leads emulsion instability by droplet aggregation. To the best of my knowledge, the influence of particle size of whey protein aggregates on the formation, properties and stability of emulsions has not been studied extensively. The objective of this study was to prepare O/W emulsions using soluble whey protein aggregates of different particle sizes at high protein concentration (6.4% w/w) and a constant oil content of 15% (w/w). The effects of crucial environmental factors such as pH, ionic strength and thermal treatment on the stability of O/W emulsions after preparation and during storage were studied to improve the understanding of potential development of real-world high protein O/W emulsions.

4.3 Materials and Methods

4.3.1 Materials

WPI (Surestart WPI 895) containing 93.54% protein (Table 4.1) was obtained from Fonterra Co-operative Ltd. (Auckland, New Zealand) and used to prepare water soluble whey protein aggregates for their use as an emulsifier. Melrose MCT Pro-rapid oil was obtained from Melrose Health, Australia and used as an oil phase in emulsions (for its product specifications, please refer to Appendix). It has been indicated by a few previous studies that some natural bioactives like curcumin have better solubility in MCT oil (Araiza-Calahorra & Sarkar, 2019; Ma et al., 2017). Nile red was used as a food colourant which was of analytical grade and obtained from Sigma Aldrich. All other chemicals used in the experiment were of analytical grade. De-ionised water was also used during the experiment.

Table 4.1 Chemical composition of Surestart WPI 895

COMPONENT	VALUE (% w/w)
Protein (6.38 x N) as is	93.54
Fat	0.35
Moisture	4.58
Lactose monohydrate	0.2
pH (@ 5% TS 20°C	6.9
Calcium (ppm)	40
Sodium (ppm)	623

Source: *WPI 895 COA, COA number - 85405089-10-1*

4.3.2 Preparation of soluble whey protein aggregates

As described in Section 3.3.2, a stock solution of WPI (20% w/w) was prepared initially and then the WPI solutions (8% w/w) with three different pH levels (6.9, 6.4 and 6.0) were prepared and stored at 4°C until use. To prepare soluble whey protein aggregates, individual whey protein solutions at three pH levels (6.9, 6.4 and 6.0) were transferred into glass bottles with a membrane screw cap and heated in a water bath maintained at 85°C for a duration of 25 min. After the heat treatment, the WPI solutions were quickly cooled under flowing tap water and kept in an ice bath (10°C) for 20 min and then stored at 4°C for 24 hr before its use.

The solution containing whey protein aggregates was not centrifuged before preparation of O/W emulsions. The choice of pH, heating temperature and duration of heating was based on the previous work on the formation of soluble whey protein aggregates described in Chapter 3. These process parameters provided the higher yield of soluble whey protein aggregates with the desired particle size.

4.3.3 Preparation of O/W emulsions

The WPI solutions containing soluble protein aggregates were warmed at 20°C for 2 hrs prior to their use in making emulsions. For the preparation of emulsion samples containing 15% oil and 6.4% protein, Melrose MCT Pro-rapid oil (15% w/w) and WPI solution (85% w/w) were combined and blended first by a high shear mixer and then by a two-stage high pressure homogeniser. Nile red (food grade colourant) was added to the oil phase at a concentration of 0.04% w/w prior to making emulsions. First, a coarse O/W emulsion was prepared with high shear mixer (Silverson L5, Silverson, UK) at 8,000 rpm for 5 min. Then, a fine emulsion was prepared by passing the coarse emulsion five times through the two-stage high pressure homogeniser (APV 2000, SPXFLOW, Denmark) (first stage at 500 bar and second stage at 50 bar). The O/W emulsion with the same concentration of oil and whey protein was also prepared using non-heated WPI solution (maintained at pH 6.9) and was treated as a control sample. After preparation, the O/W emulsions were stored at 4°C until further analysis.

Table 4.2 Experimental conditions used for the formation of O/W emulsions with soluble whey protein aggregates formed by heat treatment and at three different pH levels.

Emulsion sample code	Initial WPI soln (% w/w)	Eff. protein conc.[a] (% w/w)	pH	Temp (°C)	Heating time (min)	Emulsifier code	Oil content (% w/w)
WA 6.9	8	6.4	6.9	85	25	**SA 6.9**	15
WA 6.4	8	6.4	6.4	85	25	**SA 6.4**	15
WA 6.0	8	6.4	6.0	85	25	**SA 6.0**	15
WPI	8	6.4	6.9	Non - heated		**nWPI**	15

**Effective protein concentration in the emulsion which is the actual whey protein concentration calculated based on the WPI containing 93.54% and continuous phase of 85%.*

The whey protein aggregate dispersions as well as the O/W emulsions were prepared at least in duplicate. The relevant sample codes for designating the O/W emulsions (WA6.9, WA6.4, WA6.0 and WPI) stabilised by whey protein aggregates (SA6.9, SA6.4, SA6.0) and native whey protein (nWPI) as emulsifiers are shown in **Table 4.2**.

4.3.4 Determination of oil droplet size of O/W emulsions

The particle size of oil droplets in emulsions was measured by dynamic light scattering (DLS) technique using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, England) which is based on the measurement of Brownian motion of the particles (Analytical, 2013). In a sufficient dilute solution, the speed of Brownian motion of the particle is based on size of the particles. The correlation functions built-in equipment software provides particle size distribution data. While the fundamental size data from the equipment is based on intensity, it is converted to volume and number distribution based on Mie theory. The Zetasizer Nano ZS used is equipped with helium/neon laser with a wavelength of 632.8 nm which measures the light scattered by the sample at a backscattering angle of 173^{o}. The refractive index (RI) for emulsion droplets and dispersion medium (water) used was 1.456 and 1.33, respectively. The particle size of soluble whey protein aggregates and emulsion droplets stabilised by whey protein aggregates was analysed by using disposable cuvettes as sample cells. The protein dispersions and emulsion samples were diluted at a ratio of 1:100 and 1:500, respectively with de-ionised water before the measurement of particle and droplet size. The results of particle and droplet size measurement were expressed as Z-average which is based on intensity distribution and the size distribution of particles was based on volume frequency.

4.3.5 Determination of ζ-potential of O/W emulsions

The ζ-potential of emulsion samples was measured based on electrophoretic mobility using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, England). Before the measurement of zeta-potential, the samples were loaded into disposable cuvettes with folded capillary (DTS1070). A protein stabilised emulsion contains oil droplets which carry electrical charge which changes according to the pH of O/W emulsion. Under an electric field, the particles of oil droplets are attracted towards an opposite electrode. This movement is termed as electrophoresis. The equipment calculates ζ-potential by measurement of electrophoretic mobility (Analytical, 2013). Emulsion samples were diluted to a ratio of 1:1000 with de-ionised water before measurement to avoid multiple light scattering.

4.3.6 Determination of storage stability of O/W emulsions

Aliquots of O/W emulsion samples (25 ml) was placed in a glass vial with a screw cap and stored at 4°C for 1, 4, 7, 10 and 21 days. The particle size and ζ-potential of emulsions during storage were measured using Malvern Zetasizer Nano ZS as described in the above. Storage stability of emulsions was assessed based on change of droplet size and ζ-potential during storage.

4.3.7 Rheology of O/W emulsions

The viscosity of O/W emulsions was measured at 4°C by using a rheometer (AR 550, TA instruments) with a parallel plate geometry of 40 mm diameter (Erçelebi & Ibanoğlu, 2009; Leiva & Geffroy, 2018). A gap of 250 µm was maintained between the plates. A pre-shear duration of 60 sec and a measurement duration of 120 sec were used during the measurement cycle. A frequency range of 1 to 40 Hz was used during the process.

The curves obtained from experimental data were fitted into a power law model (Boutin et al., 2007; Roullet et al., 2019).

$$\tau = K * \gamma^n$$

Where τ is shear stress (Pa), γ is shear rate (s^{-1}), K is flow consistency index and n is flow behaviour index (dimensionless).

4.3.8 Effect of change in pH of O/W emulsions

The samples of each O/W emulsion were adjusted to pH 2, 3, 4, 5, 6 and 7 using 6 M HCl or 2 M NaOH to determine the effect of pH change by measuring the size of oil droplets using Zetasizer Nano ZS. The emulsion samples with different pH levels were also transferred to glass vials for visual observation of changes in the stability of emulsions.

4.3.9 Effect of change in ionic strength of O/W emulsions

The effect of different concentrations of $CaCl_2$ on the stability of O/W emulsions was determined. A final ionic strength of 5, 10, and 20 mM of $CaCl_2$ was achieved for each O/W emulsion by adding a suitable amount of 1 M $CaCl_2$ solution. The particle size of emulsion oil droplets was then measured by using Zetasizer Nano ZS to assess the effect of ionic strength on the emulsions. Also, a portion (2 g) of each of the resulting emulsion samples was transferred to a petri dish to assess their visual appearance to understand the rheology and presence of lumps on account of flocculation

4.3.10 Effect of thermal treatment of O/W emulsions

In order to measure the effect of thermal treatment on O/W emulsions, an aliquot (5 ml) of each O/W emulsion was placed in a glass vial with a flat screw cap (DWK Life Sciences, Germany). The glass vials were immersed in a water bath maintained at 85°C. It took 125 sec for the emulsions to reach 85°C and then the emulsions were heated for a duration of 10, 20 and 25 min. Immediately after heating, the emulsions were quickly cooled under flowing water and kept in an ice bath for 20 min and further stored at 4°C. The emulsions were visually checked for phase separation (e.g. creaming or precipitation). The presence of red dye in the oil phase provided a suitable visual marker for phase separation (if any). The droplet size of emulsions was measured by using Zetasizer Nano ZS to assess changes in the properties of emulsions due to thermal treatment. The particle size measurement of oil droplets in emulsions was conducted one day after thermal treatment of emulsions. The particle size of some emulsion samples was measured using Malvern Mastersizer 2000 because of the presence of very large oil droplets which could not be measured by Zetasizer Nano ZS.

4.3.11 Statistical Analysis

All emulsion samples were prepared in duplicate. All the measurements of samples described in the above were conducted at least in duplicate for each sample from at least duplicate experiments. The results obtained are presented as mean values ± standard deviation. A one-way analysis of variance (ANOVA) was carried to determine the significance of treatment effect at a significance level ($P \leq 0.05$).

4.4 Results and Discussion

4.4.1 Particle size and ζ-potential of O/W emulsion oil droplets

Initially, water soluble whey protein aggregates with different particle sizes were prepared at different pH levels (6.9, 6.4 and 6.0) by heat treatment of WPI solution (8% w/w) at 85°C for 25 min in order for their use as emulsifiers in preparing O/W emulsions. It should be mentioned that the heat-treated WPI solutions prepared in Chapter 4 were used as it is in the preparation of emulsions without centrifugation for the removal of water insoluble large protein aggregates. Among the heat-treated WPI solutions at three different pH levels, the presence of much larger whey protein aggregates was evident from the turbidity of whey protein aggregate dispersion formed at pH 6.0 (SA 6.0) compared to the other whey protein aggregate dispersions at pH 6.9 and 6.4 (SA 6.9 and SA 6.4) and the native WPI solution (nWPI) (**Figure 4.1A**). The particle size of native whey protein and whey protein aggregate emulsifiers measured as Zeta average diameter was 6.8 nm (nWPI), 46.1 nm (SA 6.9), 51.4 nm (SA 6.4) and 133.8 nm (SA 6.0). **Figure 4.1B** shows the particle size distribution (PSD) of the WPI solutions containing native whey protein and soluble whey protein aggregates. The results indicated that the PSDs were all unimodal although the pH 6 sample (SA 6.0) had a minor additional peak. The PSD for native whey protein (nWPI) sample was relatively narrower compared to the other samples.

As indicated above, the native whey protein (nWPI) and heat induced whey protein aggregates (SA6.9. SA6.4, SA6.0) were used as emulsifiers in this experiment to prepare O/W emulsions containing 6.4 % protein and 15% oil. All four emulsions were fluid and did not show any sign of phase separation even after centrifugation at 3000 rpm for 15 min (**Figure 4.2**). In the previous experiment (Chapter 3), it was observed that the dispersion obtained after heating 8% (w/w) WPI solution at 85°C for 25 min contained approx. 21.5% insoluble aggregates or aggregates prone to precipitation. WA 6.0 in this experiment was prepared without the removal of larger aggregates prone to precipitation. Homogenisation pressure and multiple cycles used in the preparation of emulsion were assumed to be sufficient to reduce the size of insoluble aggregates based on earlier work on reduction on the particle size of whey protein microgel (Sarkar et al., 2016) and “derivatised” whey protein aggregates

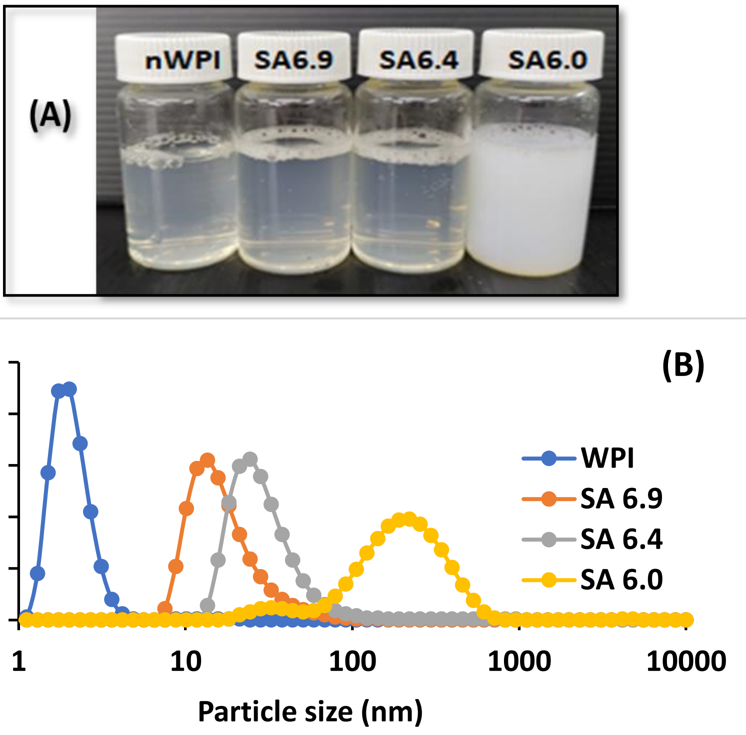


Figure 4.1 (A) Visual appearance of WPI solution (with no heat treatment) and WPI solutions (pH 6.9, 6.4 and 6.0) with different sizes of whey protein aggregates prepared by heat treatment (85°C for 25 min). SA 6.0 appears more turbid on account bigger particle size of 133.8 nm and **(B)** Particle size distributions of different whey protein particles used as emulsifiers

(Hudson et al., 2000). There was no insoluble precipitate observed visually on centrifuging the emulsions as shown in **Figure 4.2B**.

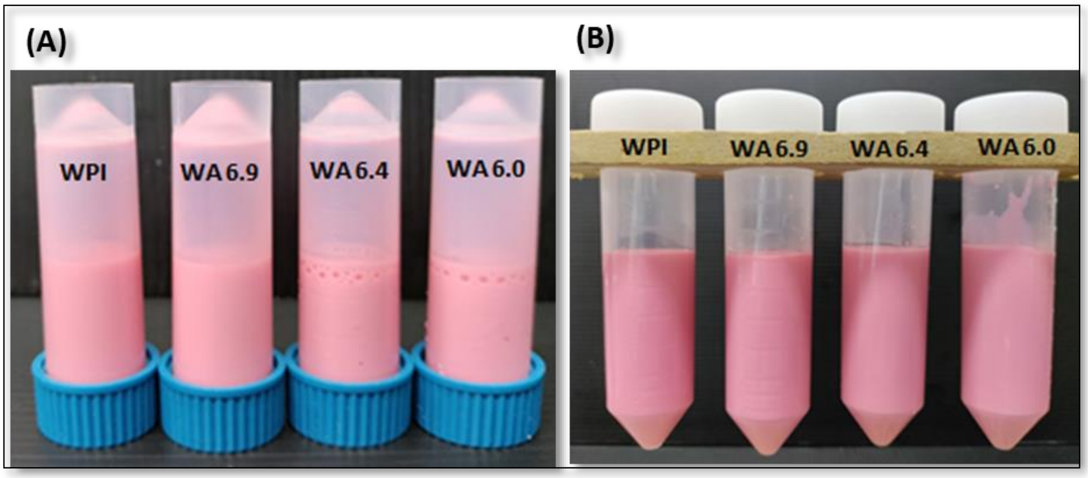


Figure 4.2 (A) O/W emulsions after 1 day of preparation. All four emulsions are fluid without any visible phase separation and **(B)** O/W emulsions observed after centrifuging at 3000 rpm for 15 min at 4°C

The characteristics (particle size and zeta potential) of four different O/W emulsions were measured one day after the preparation of emulsions which are summarised in **Table 4.3**. The oil droplet size of all emulsions was measured by using dynamic light scattering (DLS) technique. The results of droplet size were expressed in terms of Z-avg. diameter (Table 4.3). In case of the PSD curves, it was expressed as a frequency distribution based on the volume mean diameter (D_{43}) (Figure 4.3).

The Z-avg. diameter of O/W emulsions measured on Day 1 is shown in **Table 4.3.** The results indicate that the oil droplet size was significantly affected by the particle size of emulsifiers ($P \leq 0.05$). It was 162.2 nm (WPI), 219.2 nm (WA 6.9), 249.2 nm (WA 6.4) and 285.1 nm (WA 6.0). While there have not been any previous studies on the use of soluble whey protein aggregates at pH < 7, the oil droplet size confirms well with the previous studies on whey protein aggregates obtained at varying concentrations (0.1 to 8% w/w) (Boutin et al., 2007; Zhou et al., 2020) and at heat treatment of 80-95°C (Dybowska, 2011; Knudsen et al., 2008; Sobhaninia et al., 2016). Each of the earlier mentioned studies showed that the size of oil droplets increased with the particle size of aggregates. A study reported by Boutin et al. (2007) showed a droplet size of 430 nm when an emulsion (8% w/w protein and 15% w/w butteroil) was prepared with whey protein aggregates (47 nm) by homogenisation at 300 bar.

Table 4.3 Key physical properties of O/W emulsions stabilised by whey protein aggregates as well as native whey protein

Type of O/W Emulsion	DAY 1	
	Zeta – avg. diameter (nm)	Z-potential (mV)
WPI	162.2 ± 0.5^{a}	-45.7 ± 0.3^{a}
WA 6.9	219.2 ± 1.9^{b}	-43.0 ± 0.3^{b}
WA 6.4	249.8 ± 6.6^{c}	-40.6 ± 0.1^{c}
WA 6.0	285.1 ± 0.3^{d}	-36.3 ± 0.0^{d}

Letters a,b,c and d means statistically different ($P \leq 0.05$)

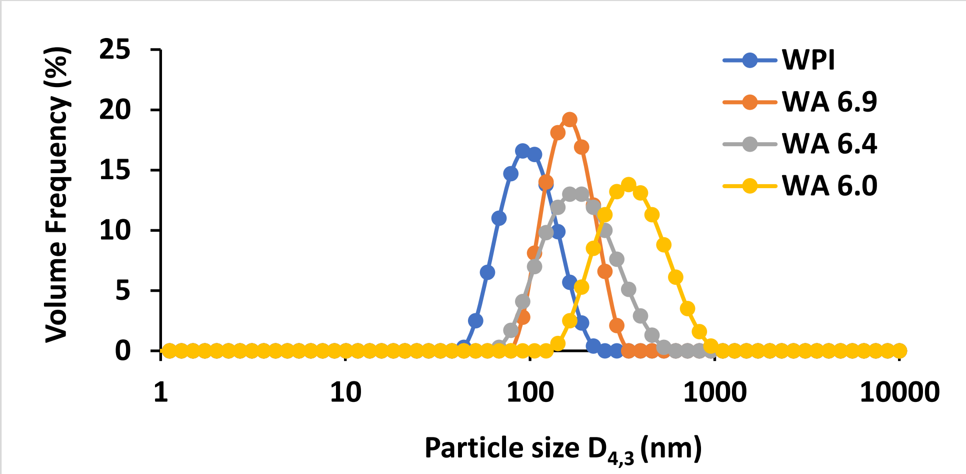


Figure 4.3 Particle size distributions of O/W emulsions measured at 4°C and at the end of 1 Day after preparation of samples.

The Zeta avg. diameter of WA 6.0> WA 6.4> WA 6.9 may indicate a formation of a much thicker interfacial layer with the use of aggregates with larger particle size. This implies that larger whey protein aggregates also appear to have formed much bigger emulsion droplets (D_{43} values of WA 6.0>WA6.4>WA 6.9) as indicated by the results of particle size distribution in **Figure 4.3**.

The higher droplet size may indicate a weaker emulsifying capacity of whey protein aggregates compared to native whey protein as indicated by earlier studies on emulsions stabilised by whey protein aggregates (Chevallier et al., 2016; Dybowska, 2011; Moro et al., 2013; Sobhaninia et al., 2017; Zhou et al., 2020). In a study conducted by (Schmitt et al., 2007), it was noted that whey protein aggregates formed at pH 6.0 had lower surface hydrophobicity than those formed at pH 7, thus resulting in a larger particle size of emulsion at pH 6.0. Besides surface hydrophobicity, the droplet size of emulsion is also influenced by speed of interfacial layer formation (Dickinson, 1999). Larger whey protein aggregates take longer to reach the interface due to higher surface viscosity and intrinsic viscosity as inferred from the previous studies on soluble whey protein aggregates (Maticorena et al., 2018; Schmitt et al., 2007; Vardhanabhuti & Foegeding, 1999).

The ζ-potential values of all emulsion samples measured on day 1 were all greater than -35 mV. This indicates that the repulsive forces acting on between oil droplets were adequate to provide the stability of emulsions against aggregation. The results also showed that the ζ-potential values were affected the particle size of whey protein aggregates. The value of ζ-potential was higher as expected in the order of WPI> WA 6.9 > WA 6.4 > WA 6.0. The ζ-potential for all emulsions was also measured on Day 21 to assess the stability of emulsions during storage at 4°C (**Figure 4.4**) which appeared to be slightly decreased.

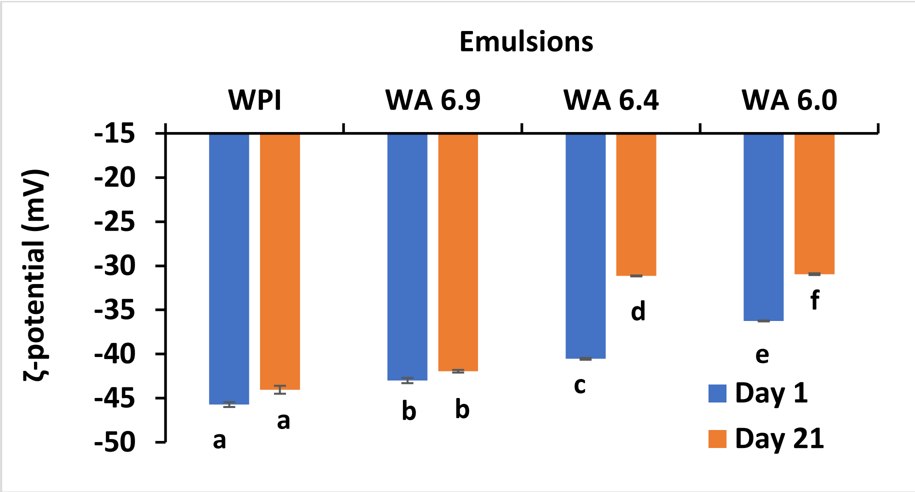


Figure 4.4 ζ-potential of emulsions measure on day 1 and day 21 after storage 4°C.

Letters a,b,c,d,e and f means statistically different ($P \leq 0.05$)

4.4.2 Storage stability of O/W emulsions

A thermodynamically unstable system like emulsion would inevitably separate into individual phases if left on its own for a longer duration of time (Dickinson, 1989; Dickinson et al., 1988). This is because emulsions can be destabilised during storage due to various mechanisms such as coalescence, flocculation, creaming, gravitational sedimentation and Ostwald ripening (Tadros, 2019). High pressure homogenisers have been found in a number of recent studies as one of the most economically viable equipment to achieve a droplet size of emulsion less than 250 nm in diameter (Karleskind et al., 1996; D.J. McClements, 2015). In the recent studies reported by several research groups (Fernández-Ávila et al., 2015; Hebishy et al., 2017; Kuhn & Cunha, 2012), the droplet size of emulsions was reduced with a use of higher pressures (> 500 bar) and multiple cycles/passes (> 3 passes) to less than 200 nm for an oil content of > 30% w/w. Fine emulsions prepared with high pressure homogenizer or microfluidizer are less prone to creaming or sedimentation in accordance with Stokes' law.

All four emulsions stored at 4°C were monitored for their stability during storage for 21 days. All emulsions remained fluid and there were no visual signs of instability during storage in terms of creaming or phase separation. Changes in the particle size of emulsion oil droplets were measured during storage at Day 1, 4, 7, 10 and 21 days (**Table 4.4A**) and ζ-potential was measured on Day 1 and 21 (**Table 4.4 B**). All emulsions had a gradual increase in droplet size through the storage period as indicated in **Table 4.4A** and **Figure 4.5**. The zeta avg values for WA 6.0, WA 6.9, WA 6.4 and WPI samples were increased by 34%, 51%, 42% and 20%, respectively. The increase in droplet size was also visible from the droplet size distributions of emulsions which appeared to be unimodal in all cases (**Figure 4.6**). The droplet size was much lower in the emulsion sample stabilised by non-heated WPI compared to those stabilised by whey protein aggregates owing to the larger size of whey protein aggregates adsorbed at the interface in conjunction with the presence of non-adsorbed large protein aggregates present in the bulk phase. For the emulsion stabilised by non-heated WPI, a gradual increase in droplet size could be due to some of those destabilisation mechanisms described above. For the emulsions stabilised by whey protein aggregates, there can be a gradual move towards higher droplet size due to protein-protein aggregation with interfacial layer (Damodaran & Anand, 1997). An increase in droplet size has been reported in some previous studies on aging emulsions stabilised by whey proteins (Kim et al., 2002; Tcholakova et al., 2006). An absence of any visible creaming during storage means no extensive droplet aggregation between oil droplets. As depletion flocculation is reversible in nature, the dilution of emulsion involved during the measurement of droplet size may have reversed the flocs (D.J. McClements, 2015).

However, this may not be the case in this study as the size was gradually increased during storage even the samples were diluted before the measurement of particle size. The emulsion samples prepared in this study seemed to be stable against creaming. The increase in apparent viscosity of emulsions resulting from the presence of whey protein aggregates in the serum phase may have prevented the emulsions from creaming. The ζ-potential values for all emulsions with whey protein aggregates were greater than -30 mV which indicates the presence of a reasonable amount of repulsive forces between droplets or flocs (**Figure 4.4 and Table 4.4**). The coalescence and flocculation stability of protein stabilised emulsions has been found to be affected by the extent of coverage of oil droplets by proteins along with the interfacial properties like surface load and thickness of layer (Delahaije et al., 2015; Dickinson & Golding, 1997a; Kim et al., 2002; Tcholakova et al., 2006). A few recent studies have indicated an increase in stability against coalescence and flocculation with increasing protein

concentration due to the efficient coverage of oil droplets (Dickinson, 1994, 1998; Euston & Hirst, 1999; Hebishy et al., 2017; McClements, 2004). High homogenisation pressure used for all four emulsions in this study led to the formation of smaller oil droplets which were believed to be efficiently covered by protein and protein aggregates due to a

Table 4.4 Storage stability of O/W emulsions during storage at 4°C. **(A)** Zeta-avg diameter measured from Day 1 to Day 21. **(B)** ζ-potential measured on Day 1 and Day 21

Type of O/W Emulsion	(A) Zeta avg diameter (nm)				
	Day 1	Day 4	Day 7	Day 10	Day 21
WPI	162.2 ± 0.5^{a}	171.3 ± 1.5^{b}	178.1 ± 2.0^{b}	175.3 ± 1.8^{b}	194.2 ± 5.5^{c}
WA 6.9	219.2 ± 1.9^{d}	262.1 ± 0.5^{e}	264.6 ± 0.6^{e}	276.8 ± 3.2^{e}	330.3 ± 0.6^{f}
WA 6.4	249.8 ± 6.6^{g}	281.1 ± 3.2^{h}	289.6 ± 1.2^{h}	297.0 ± 8.5^{h}	354.3 ± 2.5^{i}
WA 6.0	285.1 ± 0.3^{j}	294.9 ± 1.3^{k}	300.0 ± 10.6^{k}	302.6 ± 4.1^{l}	383.8 ± 0.8^{m}

Type of O/W Emulsion	(B) ζ-potential (mV)			
	Day 1	Day 4/7	Day 10	Day 21
WPI	-45.7 ± 0.3	-	-	-44.1 ± 0.4
WA 6.9	-43.0 ± 0.3	-	-	-42.0 ± 0.1
WA 6.4	-40.6 ± 0.1	-	-	-31.2 ± 0.0
WA 6.0	-36.3 ± 0.0	-	-	-31.0 ± 0.1

Superscript letters a,b,c,d,e,f,g,h,I,j,k,l and m mean statistically different ($P \leq 0.05$)

indicated an increase in stability against coalescence and flocculation with increasing protein concentration due to the efficient coverage of oil droplets (Dickinson, 1994, 1998; Euston & Hirst, 1999; Hebishy et al., 2017; McClements, 2004). High homogenisation pressure used for all four emulsions in this study led to the formation of smaller oil droplets which were believed to be efficiently covered by protein and protein aggregates due to a high concentration of protein (6.4% w/w based on 8% w/w WPI) used in this study. The enhanced creaming stability has been attributed to the formation of a thick planar gel like structure by previous studies on O/W emulsions stabilised by whey proteins/β-lg (Dickinson, 1999; Dickinson & Matsumura, 1994). The thickness of interfacial layer of emulsion droplet stabilised by whey protein/β-lg has been shown to increase due to covalent and non-covalent bonds formed between adsorbed proteins and other non-adsorbed proteins by several recent studies (Damodaran &

Anand, 1997; Fang & Dalgleish, 1997; Murray et al., 2002; Tcholakova et al., 2006). The stability of droplets formed by whey protein aggregates in this study could also be due to an increase in density of droplets due to the formation of thicker interfacial layer (Dickinson, 1992a).

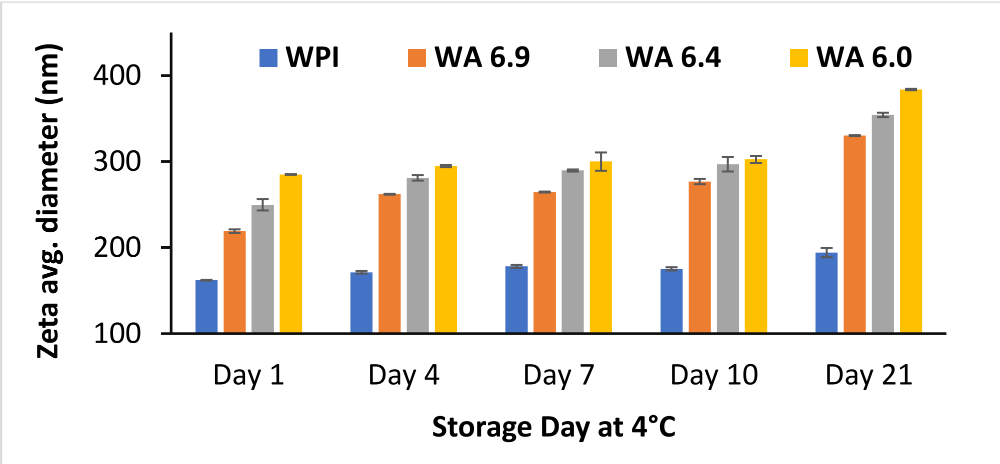
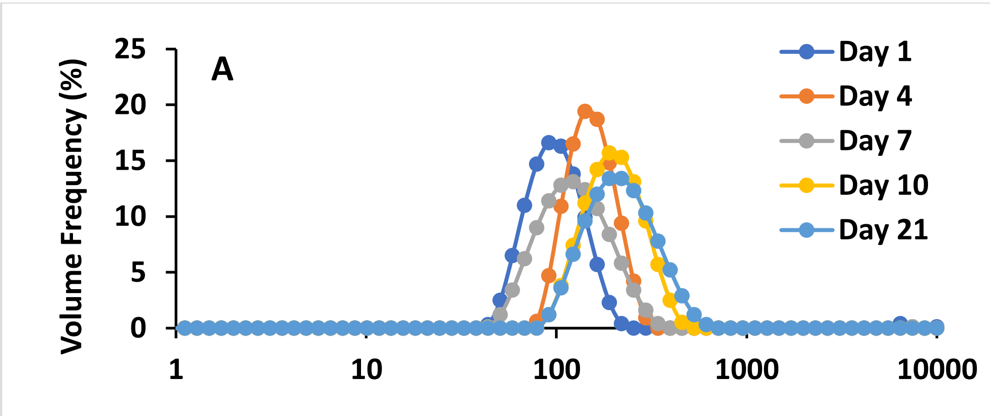
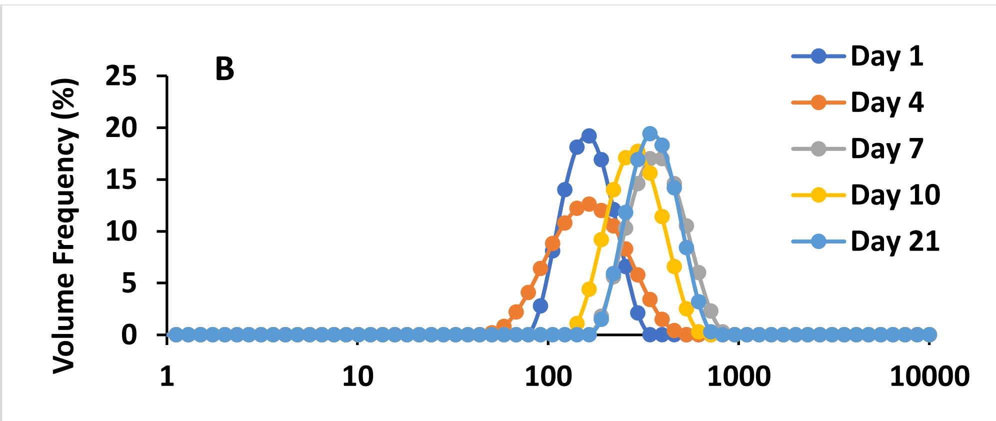


Figure 4.5 Stability of O/W emulsions during storage at 4°C measured as change in the particle size of emulsion oil droplets





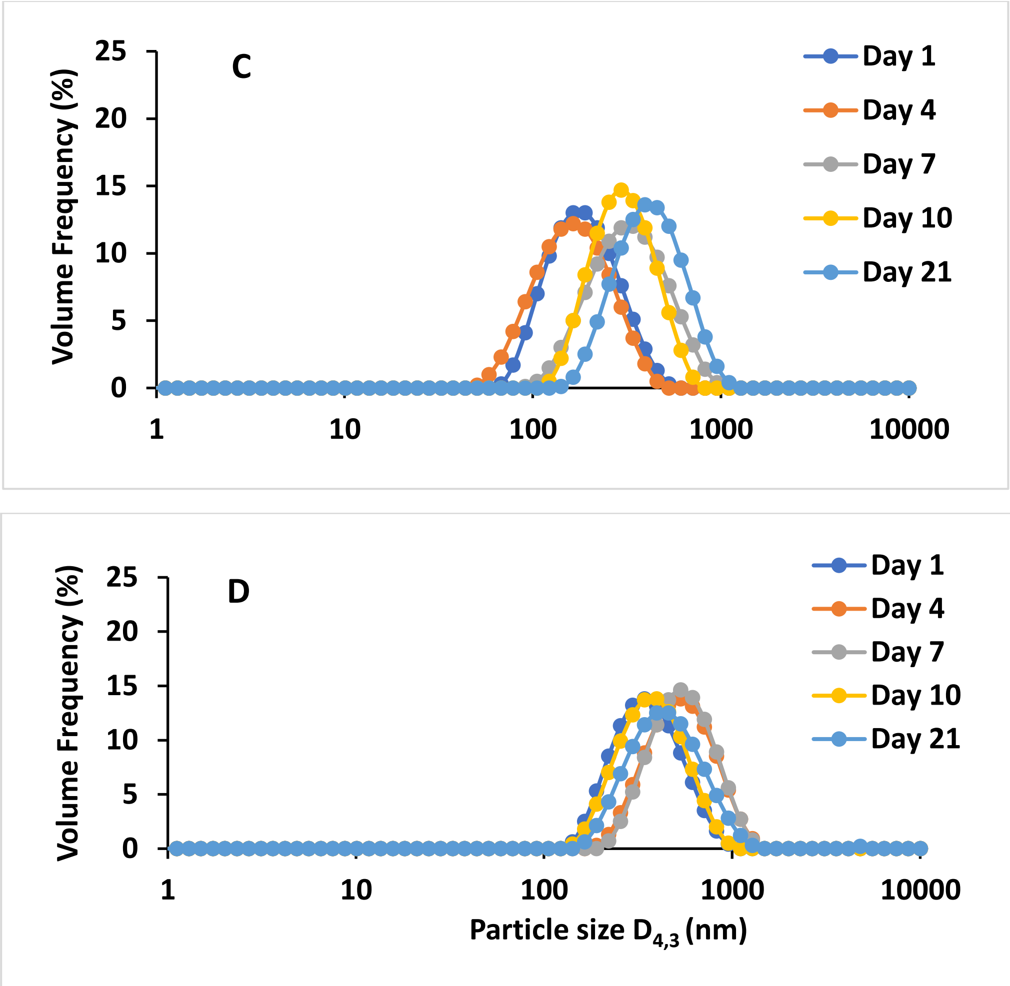


Figure 4.6 Particle size distributions of O/W emulsions measured during storage at 4°C. **(A)** WPI, **(B)** WA 6.9, **(C)** WA 6.4 and **(D)** WA 6.0.

4.4.3 Rheology of O/W emulsions

The viscosity of emulsions was measured at 20°C. The results shown in **Figure 4.7** indicate that the particle size of whey protein and whey protein aggregates had a significant effect on the apparent viscosity of emulsions ($P \leq 0.05$). The apparent viscosity of emulsions measured at a shear rate of $50s^{-1}$ is shown in **Table 4.5**. It has been noted from the literature that the viscosity measured at this shear rate provides a useful correlation to desirable sensory properties (creaminess) (Kokini & Cussler, 1983; Richardson et al., 1993). It is evident that the emulsion stabilised by non-heated whey protein exhibited a Newtonian behaviour. For the Newtonian emulsions, flow consistency index (K) is equal to the viscosity of emulsion (η) and flow behaviour index (n) = 1 (Roullet et al., 2019; Tadros, 2010). Non-Newtonian or shear thinning behaviour was observed from the emulsions stabilised by whey protein aggregates

(WA 6.9, WA 6.4, WA 6.0) with the value of n< 1 for all these emulsions. The flow consistency index (K) increased significantly with the particle size of emulsifier (non-heated whey protein and whey protein aggregates) (**Table 4.5**). The shear thinning behaviour of emulsions confirms well with the previous studies with heat induced aggregates of whey protein (Boutin et al., 2007; Eissa et al., 2014; Sobhaninia et al., 2016) and β-lg (Knudsen et al., 2008). In each of these studies, whey protein aggregates have been compared with native whey protein.

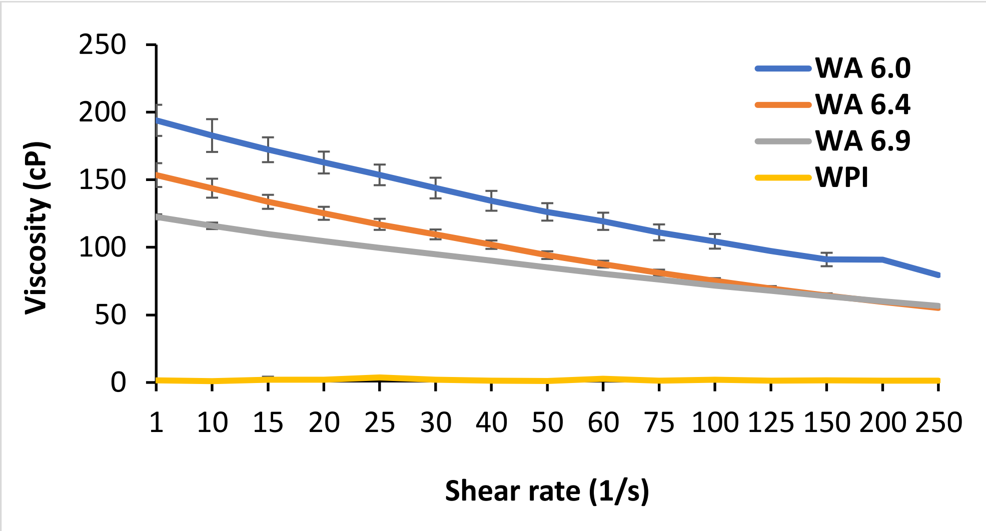


Figure 4.7 Apparent viscosity (η) of O/W emulsions measured at **20°C**

Table 4.5 Viscosity and rheology parameters based on the power law equation for all O/W emulsions. K and n represent flow consistency index and flow behaviour index, respectively. All emulsions were measured at **20 °C.**

Emulsion code	Rheology parameters			
	Viscosity (mPa-s)^x	K (Pa-s^n)	n	R^2
WPI	1.65 ± 0.2	0.002 ± 0.034^a	1	0.993
WA 6.9	85.1 ± 0.9	0.202 ± 0.034^b	0.796 ± 0.08	0.983
WA 6.4	94.2 ± 3.6	0.311 ± 0.013^c	0.700 ± 0.013	0.991
WA 6.0	135.6 ± 7.6	0.398 ± 0.034^d	0.717 ± 0.032	0.994

x Measured at shear rate of 50s^{-1}. Letters a,b,c and d mean statistically different ($P \leq 0.05$)

For the emulsions stabilised by whey protein aggregates, the amount of non-adsorbed protein aggregates was in the order of WA 6.0 > WA 6.4 > WA 6.9 because of lower emulsifying capacity of larger whey protein aggregates. In earlier studies on non-heated β-lg and β-lg (in the size range of 35 to 60 nm), it was observed that while surface hydrophobicity of whey protein aggregates was higher than non-heated WPI, emulsifying activity index was lower that non-heated WPI (Knudsen et al., 2008; Moro et al., 2013; Moro et al., 2001). The underlying reasons have not been established for such behaviour (Moro et al., 2013). The particle size of whey protein aggregates used in the preparation of emulsions for WA 6.9, WA 6.4 and WA 6.0 was 46.1 nm (SA 6.9), 51.4 nm (SA 6.4) and 133.8 nm (SA 6.0), respectively, as already described in Section 4.4.1. This appears to have led to increase in apparent viscosity of emulsions with increasing particle size of aggregates. A hydrocolloid such as gum arabic carries a rather desirable dual quality of ingredient functions as an emulsifier as well as a thickening agent (Dickinson, 2003; Garti, 1999; Ibanoglu, 2002). This has been attributed to the presence of a protein moiety attached to gum arabic (Dickinson, 2003, 2009; Randall et al., 1988). The flow consistency index of gum arabic has been shown to increase with gum concentration (Gómez-Díaz et al., 2008). In an earlier study on rheological properties of whey protein emulsions containing varying quantity of gum arabic concluded that the apparent viscosity and flow consistency index increased with the particle size and concentration of non-adsorbed gum arabic (Ibanoglu, 2002). A previous study on β-lg fibrils indicates that the length of fibrils has a significant impact on induction of depletion flocculation in emulsions (Blijdenstein et al., 2004). The results obtained in the present study confirms that size of soluble whey protein aggregates in the continuous phase has a significant impact on extent of depletion flocculation in the emulsion as indicated by rheological measurement of emulsions.

The shear thinning behaviour of emulsions stabilised by whey protein aggregates indicates a presence of depletion flocculation (Boutin et al., 2007; Dybowska, 2011; Dybowska & Krupa-Kozak, 2020). Bridging flocculation appears highly unlikely as the protein to fat ratio (1:2) used in this study is sufficiently high in each to ensure sufficient coverage of emulsions. Depletion flocculation observed in emulsions characterised by whey protein aggregates is due to the presence of non-adsorbed whey protein aggregates in the continuous phase (Boutin et al., 2007; Roullet et al., 2019). Emulsion droplets are kept separated from each other due to repulsive forces between them. In the presence of non-adsorbing polymers in the continuous phase, the gap between droplets may be vacated if the size of the polymer is much bigger than the droplet due to increase in osmotic pressure. It is thermodynamically favourable for the polymer to move away from the gap into a relatively dilute bulk phase (Dickinson, 1992b). The ensuing droplet aggregation is termed as "depletion flocculation". Droplet aggregation may not continue unhindered as the depletion flocculation is accompanied with an increase in the viscosity of continuous phase which limits further collision of droplets. Aggregated droplets or flocs in flocculate emulsions tend to occupy larger amount of water than non-flocculated droplets(D.J. McClements, 2015). The resultant effective volume fraction of droplets increases the radius of droplets (Walstra, 2002) as well as the thickness of interfacial layer (Tadros, 2010). The increase in volume fraction of droplets leads to increase the viscosity of the emulsion (Pal, 2000; Tadros, 2010). The interfacial layer formed by whey protein aggregates forms a thicker compact structure due to disulphide bond between aggregates present in the layer. Euston and Hirst (1999) has also indicated droplet- droplet aggregation promoted by "fusing of interfacial layers. The thickness of interfacial layer for emulsion would increase with particle size. Emulsions with flocculated droplets exhibit "shear thinning" behaviour or reduction in apparent viscosity when subjected to increasing shear rate due to breakdown of flocs or alignment of flocs with the shear field. Depletion interaction can be increased by increase in molar concentration of biopolymer in the continuous as well as increase in the size of the biopolymer (McClements, 2000). Emulsions stabilised by sodium caseinate or casein exhibit depletion flocculation with rise in concentration of non-adsorbed sodium caseinate (Dickinson & Golding, 1997a; Hemar et al., 2003). Colloidal particles like sodium caseinate or casein micelles have a particle size of 10 – 50 nm (De Wit, 1998; Lucey et al., 2000). Whey protein aggregates have a significant role to play on the volume fraction of the droplet (Boutin et al., 2007). Viscosity of protein dispersion has been shown to increase with protein concentration, weight and size of protein due to a larger volume fraction occupied by protein in the dispersion (Boulet et al., 1998; Bullard et al., 2009). In a previous study on volume fractions with whey protein aggregates, it was found that volume fraction was directly proportional to size of the aggregate (Alting et al., 2003; Boutin et al., 2007). The increase in apparent viscosity can clearly be attributed to an increase in particle size of emulsifiers as well as presence of non-adsorbed whey protein aggregates on account of higher WPI concentration used in the study (8% w/w).

4.4.4 Effect of change in pH of O/W emulsions

Food emulsions (acidified milk beverages, sports beverages, salad dressings, mayonnaise, soups, sauces) and emulsion gels (yogurts, cream cheese, sour cream) have a pH in the range of 3 to 6. Reduction of pH (as well as addition of minerals/salts) leads to aggregation due to screening of charges on emulsion droplets and non-adsorbed protein in the bulk phase.

The pH of each emulsion prepared was adjusted to 2, 3, 4, 5, 6 and 7 to determine the effect of pH change on the particle size and stability of emulsions. The oil droplets and non-adsorbed proteins in the original emulsions (WPI, WA 6.9, WA 6.4 and WA 6.0) prior to pH adjustments would bear a negative charge. A presence of repulsive forces was also evident from the values obtained for ζ-potential (**Figure 4.4**). The results showed that all four emulsions remained stable and in a liquid state when their pH was changed to 7.0, 6.0, 3.0 and 2.0. The droplet size measured for each of them is provided in **Figure 4.8**. All emulsions showed the highest droplet size at pH 6. The increase in droplet size for WPI, WA 6.9 and WA 6.4 as pH was reduced to 6 could be due to reduction in repulsive forces which might allow for possible adsorption of non-adsorbed proteins. In a previous study on emulsions prepared at pH 7 and pH 6 reported by Hunt and Dalgleish (1994), it was noted that the stability of

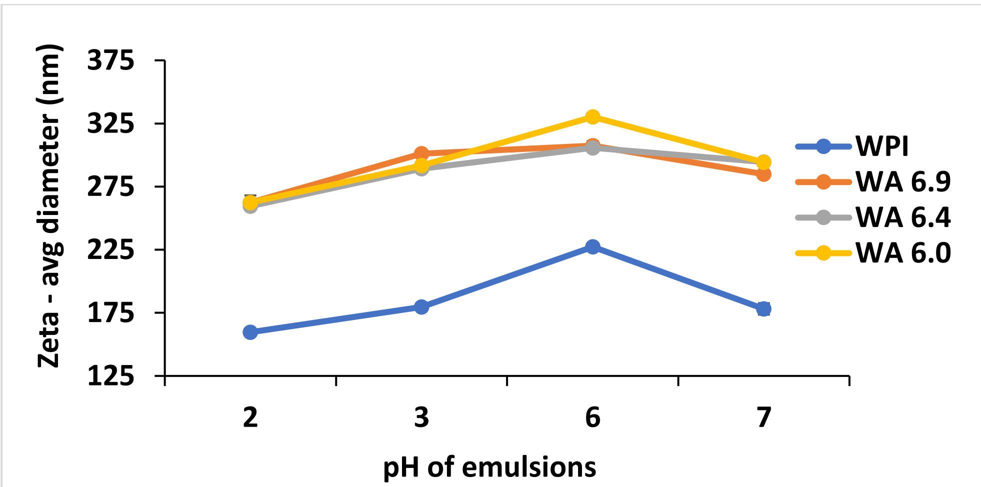


Figure 4.8 Changes in the particle size of O/W emulsions due to pH change. Emulsions were stabilised by different whey protein particles.

emulsion at pH close to the isoelectric point is due to preferential adsorption of α-la over β-lg. At pH 2 which is sufficiently far away from the isoelectric point of whey proteins, repulsive forces curbed any droplet aggregation. The smallest droplet size was observed from all the emulsions at pH 2.

However, the emulsions formed gels due to droplet aggregation when their pH was adjusted to pH 4 and 5. This was observed from all emulsions stabilised by whey protein aggregates, such as WA 6.9, WA 6.4 and WA 6.0. The emulsion stabilised by non-heated WPI formed a paste like structure (**Figure 4.9**). A number of previous studies on cold-set whey protein gels have proved that in the presence of heat treated protein aggregates, gelation of whey proteins can be achieved at protein concentrations much lower than its critical gelation concentration (Alting et al., 2000; Bryant & McClements, 1998; Doi, 1993). Gelation of protein-stabilised emulsions has been categorised as emulsion-filled gels and emulsion gels based on the structural arrangement of such soft solids. Emulsion gels are formed as emulsion droplets aggregate and turn liquid viscoelastic emulsion into soft deformable solids. Emulsion-filled gels involve emulsion droplets embedded in a gel network formed by aggregated proteins (Dickinson, 2012a; Ye & Taylor, 2009).

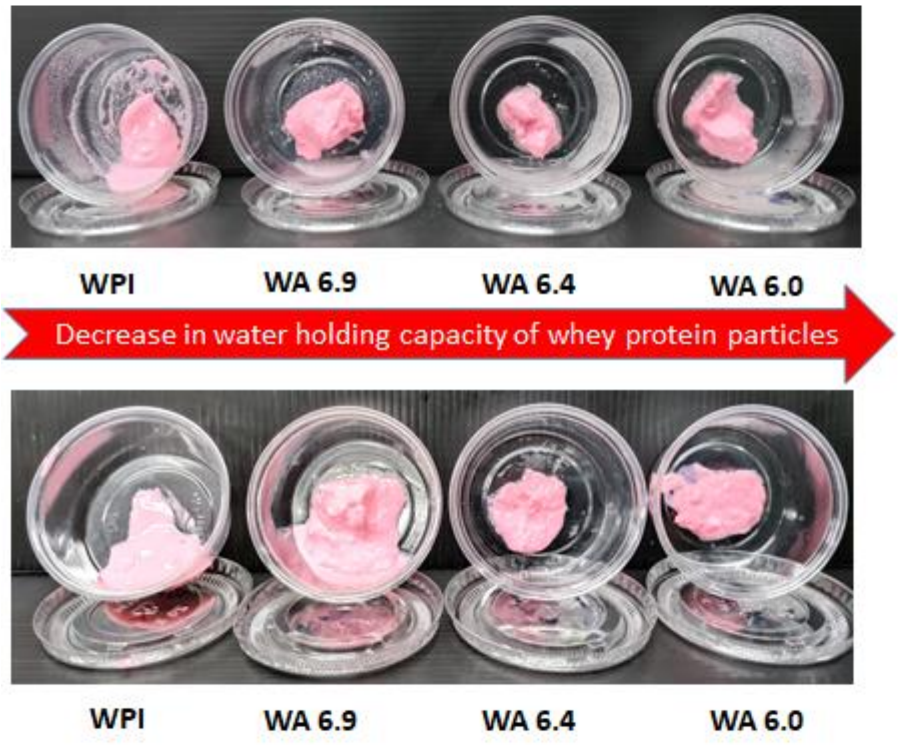


Figure 4.9 Visual observation of emulsion samples in relation to change in pH of emulsions stabilised by different whey protein particles. pH = 5 **(top)**, pH = 4 **(bottom)**

In the context of the present study, non-adsorbed proteins play a crucial role in the formation of gels. Non-adsorbed heat-treated proteins in the continuous phase have been shown to form gels by two processes – forming an extensive gel network (akin to cold-set gels) and "gluing" together emulsion (Dickinson & Chen, 1999; Hunt & Dalgleish, 1995; Ye & Taylor, 2009). Based on mere visual observation, it was difficult to conclude on the type of gel formed. However, whey protein emulsion gels formed by acidification of emulsions have been characterised by earlier studies as both emulsion-filled gels (Rosa et al., 2006)

with oil droplets behaving as "active" fillers (Dickinson & Chen, 1999) as well as emulsion gels (Boutin et al., 2007). Active fillers play an important role in the mechanical properties of the gel (Rosa et al., 2006; Sok Line et al., 2005). For WA 6.9, WA 6.4 and WA 6.0, the visual difference in the properties of emulsion gels clearly supports that higher concentration of non-adsorbed heat-treated protein aggregates leads to increasing the mechanical properties (storage modulus, hardness, and stiffness) of gels (Rosa et al., 2006; Sok Line et al., 2005; Ye & Taylor, 2009). The stiffness and syneresis of emulsion-filled gels, observed visually, increased with increasing particle size of whey protein aggregates (WA 6.0 > WA 6.4 > WA 6.9). In previous studies on cold-set whey protein gels induced by pH, the strength and hardness of gel increased with the extent of disulphide -thiol interaction and aggregate size (Alting et al., 2000; Bryant & McClements, 2000; Ju & Kilara, 1998; Mleko, 1999). In a recent study on emulsion gels prepared by whey protein aggregates at oil content of < 20% at pH 4 with the help of glucono-δ-lactone, it was noted that the concentration of whey protein aggregates had a significant effect on the characteristics of emulsion gels (Boutin et al., 2007). In an similar study reported by Mantovani et al. (2016), it was observed that the emulsions stabilised by whey protein aggregates formed self-supporting gels only in the presence of non-adsorbed whey protein aggregates.

Emulsion gels formed at pH 4 appeared weaker compared to those formed at pH 5. This compares well with a recent study on the effect of pH on the structural properties of whey protein emulsion gels in which it was observed that hardness and elasticity of gels at pH 5.2 was greater than at pH 3.9 (Mantovani et al., 2016). In the case of WA 6.9, it appeared that the emulsion gel formed was much weaker at pH 4 when compared with self-supporting gel formed at pH 5. As mentioned earlier, it could possibly be due to the presence of much lower non-adsorbed proteins (SA 6.9) available in the bulk phase as more protein aggregates might have accumulated at the interface due to higher surface hydrophobicity of aggregates (Schmitt et al., 2007).

Previous studies on emulsions stabilised by non-heated noted the formation of a high viscous paste due to droplet aggregation at pH 4.5 to 5.5 while the emulsion remained fluid at pH 4 (Demetriades et al., 1997). The concentration of WPI used in that study was 1% w/w. As seen in **Figure 4.9**, the emulsion formed by non-heated whey protein (WPI) formed a highly viscous fluid at pH 4 and a high viscosity paste at pH 5. The role of higher protein content and α-la (pI = 4.2) could be responsible for droplet aggregation at pH 4.

Preliminary studies on pH change of WPI solutions

Along with emulsions, change of pH was also studied for solutions containing non-heated WPI and soluble whey protein aggregates. The pH of these solutions was changed to pH = 7,6,5,4,3 and 2 and visual observations were noted as indicated in **Figure 4.10.** The solution containing non-heated WPI (nWPI) showed some precipitation of protein at pH = 5 which is closer to isoelectric point of WPI (≈5.1) (**Figure 4.10 A**). All solutions containing soluble whey protein aggregates formed gels at pH = 5 and 4. (**Figure 4.10 B, C and D**). The gels were turbid at pH = 5 compared to pH = 4 which could indicate much firmer gel network. The solution containing larger soluble whey protein aggregates (SA 6.0) formed turbid gels at pH = 5 and 4 which could indicate much rigid and firm protein gel. These results obtained using whey protein solutions confirm well with results obtained on changing pH of O/W emulsions (WPI, WA 6.9, WA 6.4 and WA 6.0).

4.4.5 Effect of change in ionic strength of O/W emulsions

Several nutritional beverages (emulsions) containing whey proteins are often fortified with minerals/salts (Keowmaneechai & McClements, 2006). Typical levels of calcium in beverages would range from 1 mM to 15 mM (Grestner, 2003; Hansen et al., 1996; Liebrecht & Phillips, 2000). An addition of a salt (e.g., NaCl, $CaCl_2$, KCl) to an emulsion at a constant pH screens repulsive forces between emulsion droplets and proteins in the bulk phase. Addition of salt to protein stabilised emulsions reduces the quantum of repulsive forces between emulsion

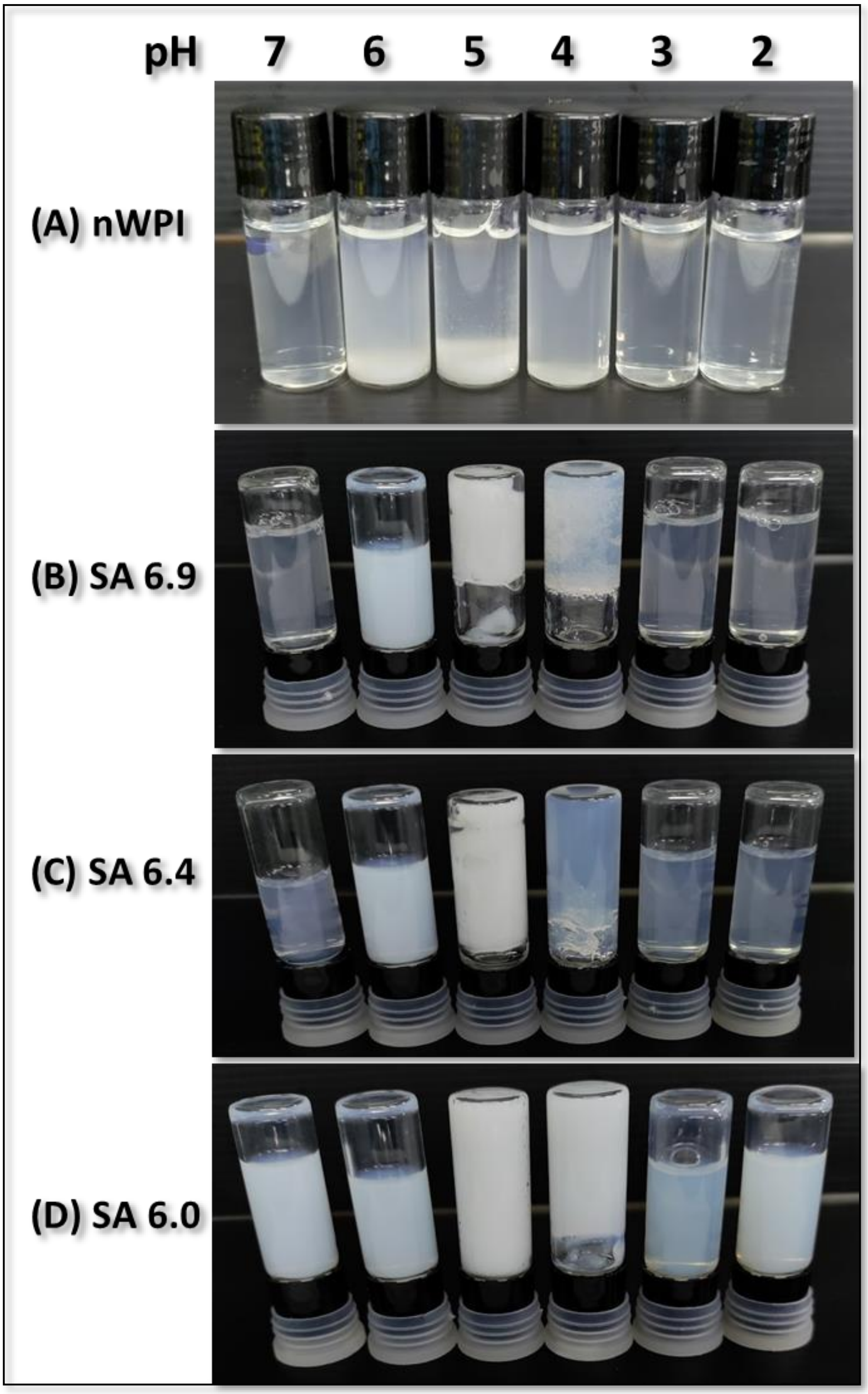


Figure 4.10 Effect of change of pH from 7 (Left) to 2 (Right) for WPI dispersions containing soluble whey protein aggregates **(A)** nWPI, **(B)** SA 6.9, **(C)** SA 6.4 and **(D)** SA 6.0

droplets and non-adsorbed protein in the continuous phase. The screening of electrical charges leads to emulsion destabilisation by extensive droplet aggregation due to a decrease or loss of electrical net charge (Demetriades et al., 1997; Dickinson & Galazka, 1991; Hunt & Dalgleish, 1994). Flocculation is considered among the chief reason for such aggregation as the flocculated oil droplets become fused resulting in coalescence leading to aggregation (Dickinson, 2010). As depicted in **Figure 4.11**, all four emulsions underwent significant droplet

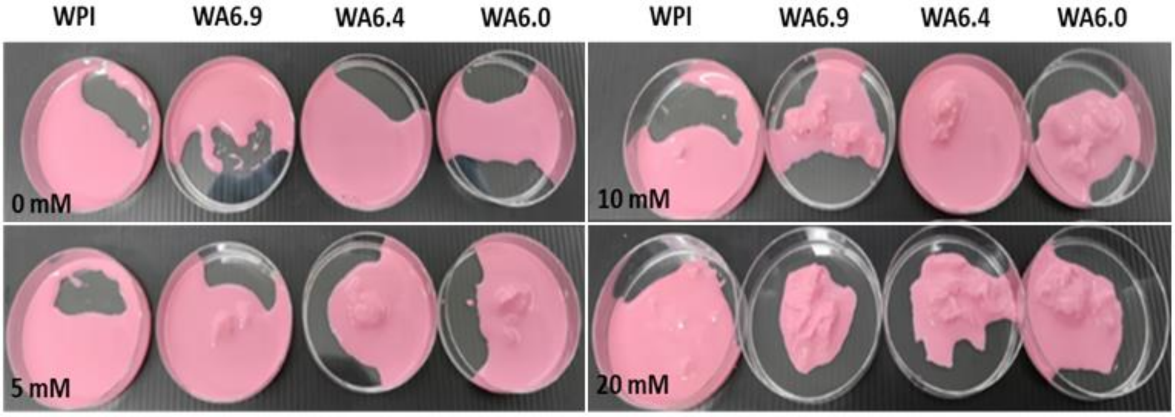


Figure 4.11 Effect of change in $CaCl_2$ concentration (0, 5, 10 and 20 mM) on the O/W emulsions stabilised by different whey protein particles.

aggregation at a $CaCl_2$ concentration of > 5 mM. The calcium content in Surestart 895 (WPI powder) used in this experimental work contained only 40 ppm of calcium and it was thus assumed that it did not have a significant effect on calcium induced droplet aggregation in emulsions. The O/W emulsion stabilised by native whey proteins (WPI) formed visible flocs at $CaCl_2 \geq 10$ mM. Mild droplet aggregation in the WPI solution without heat treatment could be identified at $CaCl_2$ 5 mM based on the results of particle size (data not shown) and particle size distribution as presented in **Figure 4.12**. At lower protein concentration (< 5% w/w) in emulsion, an extensive droplet aggregation has been shown at $CaCl_2 \geq 3$ mM (Keowmaneechai & McClements, 2006; Kulmyrzaev et al., 2000; Ye & Singh, 2000). It has also been shown in a previous study on WPC that droplet aggregation was significant only at $CaCl_2 \geq 10$ mM when WPC concentration was increased from 0.5% w/w to 3% w/w (Ye & Taylor, 2009) Droplet aggregation due to Ca^{++} occurs due to the formation of calcium linkages between emulsion droplets and non-adsorbed whey protein particles in the dispersed phase

(Dickinson, 2010). At higher protein concentration, more $CaCl_2$ would be needed to induce a visually significant droplet aggregation.

All emulsions stabilised by whey protein aggregates (WA 6.9, WA 6.4 and WA 6.0) formed visible flocs at $CaCl_2$ 5mM. It has been shown in previous studies that whey protein aggregates possess more calcium binding sites as compared to native proteins (Kharlamova et al., 2018). Hence, aggregation of emulsion droplets and larger whey protein aggregates have been formed in these emulsions at a much lower $CaCl_2$ concentration (Kharlamova et al., 2018).

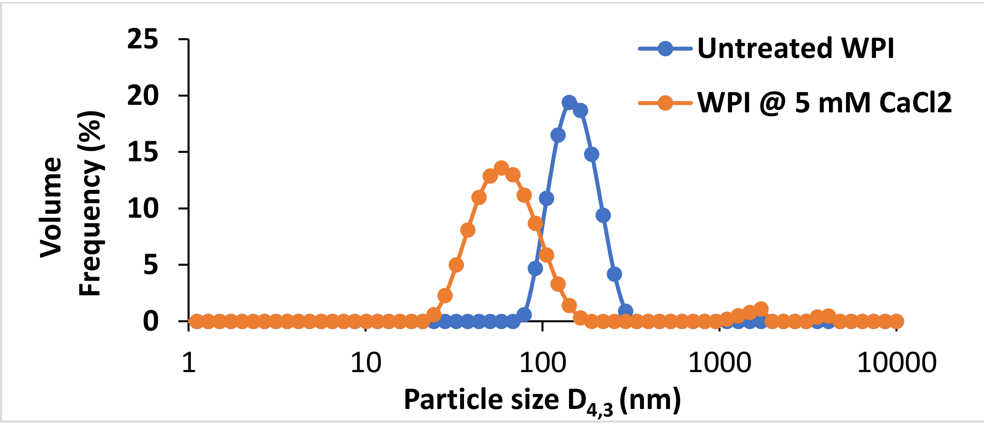


Figure 4.12 Effect of addition of $CaCl_2$ on the particle size distribution of emulsions stabilised by non-heated whey protein (WPI) solution.

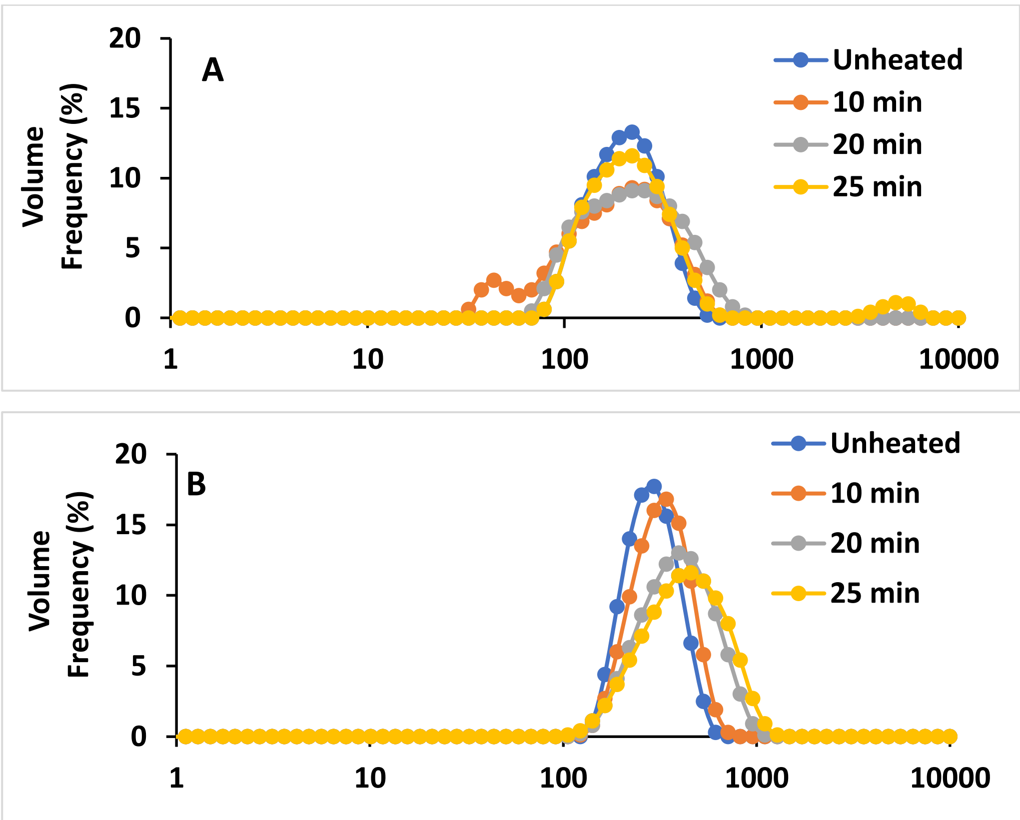
4.4.6 Effect of thermal treatment of O/W emulsions

Thermal treatment is often used in the food industry to ensure a longer shelf life by inhibition of microbial growth. Emulsions stabilised by a heat labile, globular protein like whey protein are prone to destabilisation (coalescence and flocculation) due to thermal treatment (Deeth, 2020; Wijayanti et al., 2019). Assuming thermal stability of oil, thermal stability of protein stabilised emulsion would primarily depend upon stability and changes in protein at the interfacial layer and the bulk phase. While experiment in this chapter dealt with emulsion stability affected by native or protein particles at constant oil content, the effect of oil content and cascading effect on interfacial and bulk proteins on emulsion stability was explored in the next chapter.

All emulsions were found to be fluid and no visible flocs or layer separation was observed after heating each of them at 85°C for 10, 20, and 25 min. For WA 6.9, WA 6.4 and WA 6.0

emulsions, non-adsorbed protein aggregates were reheated at similar heating conditions that had initially been applied to the WPI solution. The particle size distribution of oil droplets in emulsions (WPI, WA 6.9, WA 6.4, and WA 6.0) with and without heat treatment is shown in **Figure 4.13** which shows a gradual increase in size as the heating progressed. A gradual increase in the droplet size (**Table 4.6**) occurred possibly due to thickening of interfacial layer as more non-adsorbed proteins (both native and whey protein aggregates) formed disulphide bonds with proteins at the interface (Damodaran & Anand, 1997; Dickinson, 1992a; Monahan et al., 1996). The nature of non-adsorbed proteins in the bulk phase in the emulsion can influence the characteristics of droplets upon thermal treatment of emulsions.

For an emulsion stabilised by non-heated WPI, one could detect a minor presence of droplet aggregation at the end of 10 min. Thermal treatment of emulsion led to aggregation of non-adsorbed WPI. As noted in the results obtained in the earlier study (Chapter 3), a thermal treatment of WPI solution (at pH = 6.9) at 85°C for more than 2.5 min. is sufficient to form whey protein aggregates in the size range of 40 to 50 nm. The presence of droplet aggregation could have occurred due to depletion flocculation induced by these non-adsorbed aggregates. Although very few in number (based on frequency), droplet aggregates of bigger size (> 2 μm)



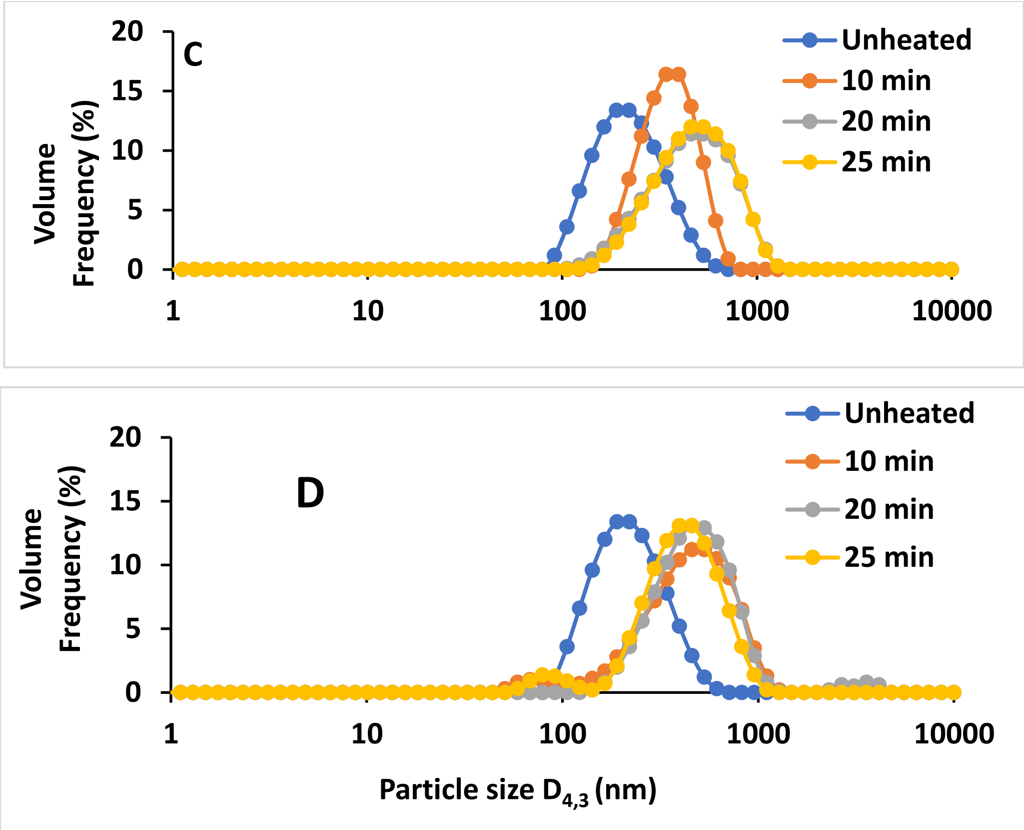


Figure 4.13 Particle size distribution of emulsions without and with heat treatment (85°C for 10, 20 and 25 min). **(A)** WPI, **(B)** WA 6.9 **(C)** WA 6.4, and **(D)** WA 6.0.

were detected at the end of 25 min. The O/W emulsions stabilised by non-heated WPI have been shown in the literature to be stable to thermal treatment in the range of 80 to 95°C for a duration of 30 min (Damodaran & Anand, 1997; Fang & Dalgleish, 1997; Monahan et al., 1997). However, the stability of emulsions to thermal treatment has been shown to be influenced by a protein to oil ratio (Ye & Taylor, 2009). It has been shown in a previous study on O/W emulsions stabilised by whey proteins that further accumulation of protein stops at protein concentration of > 3% w/w (Euston & Hirst, 1999). The study was conducted using 1-8% (w/v) of WPC and oil content of 20%. On account of high protein concentration (8% w/w WPI) used in this study, one could assume a sufficient amount of non-adsorbed proteins present in the continuous.

For WA 6.9, WA 6.4 and WA 6.0, non-adsorbed protein aggregates were reheated at similar heating conditions. In a previous study on cold-set gels formed by whey proteins, it was found that in a two-step heating process at 80°C, the size of aggregates is increased in the second heating cycle as protein concentration is increased (Mleko, 1999). Possibly, aggregates behaved as "seeds" or aggregation sites for further aggregation. The nature of droplets would be affected by interaction between droplets influenced by non-adsorbed protein aggregates. In an earlier study on the role of non-adsorbed whey protein aggregates obtained on thermal heating O/W emulsions, it was found that non-adsorbed protein aggregates could interact with droplets by two mechanisms (Euston et al., 2000). The droplets could accumulate onto the existing interface or act as a "gluing" or "fusing" agent for the droplets. The study could not establish dominance of either mechanism on heating O/W emulsions stabilised by whey proteins.

Table 4.6 Effect of heating O/W emulsions at **85 °C** for different times (10, 20 and 25 min) stabilised by different whey protein particles. Zeta-avg diameter (or hydrodynamic diameter) of O/W emulsion before and after thermal treatment.

Type of Emulsion	Zeta-avg diameter (nm)			
	Unheated	**85°C/10 min**	**85°C/20 min**	**85°C/25 min**
WPI	175.0 ± 1.8	184.8 ± 1.1	189.1 ± 1.1	196.1 ± 1.8
WA 6.9	276.8 ± 3.2	288.4 ± 4.8	306.4 ± 0.1	309.8 ± 0.3
WA 6.4	287.3 ± 1.2	301.0 ± 6.1	305.7 ± 2.5	319.0 ± 2.8
WA 6.0	302.6 ± 4.1	311.0 ± 2.5	320.3 ± 6.8	329.7 ± 1.9

Emergence of much larger flocs in heated WA 6.0 which were not detected in unheated emulsions indicates rise in droplet aggregation, possibly by fusing of droplets by non-adsorbed whey protein aggregates. There was no droplet aggregation detected for WA 6.9 and WA 6.4. It has been noted in recent studies by Dybowska (2011) and Dybowska and Krupa-Kozak (2020) that emulsions stabilised by whey protein aggregates formed at near neutral condition (whey protein particles of 122 to 342 nm) have better emulsion stability than emulsions stabilised by native proteins. The emulsifiers used for both emulsions, SA 6.9 and SA 6.4, had smaller particle size of 46.1 nm and 51.4 nm, respectively.

In a recent study on dispersion of soluble whey proteins aggregates of 49 nm which was formed on heating at 90°C for 15 min was reheated at the same condition on dilution (Ryan et al., 2012). A solution of native protein was also treated at the same condition for comparison in change in viscosity of the resulting dispersion. Re-heating of whey protein aggregate dispersion did not lead to any change in viscosity. The underlying mechanism for improved thermal stability of soluble whey protein aggregate remains unidentified and needs to be further investigated.

4.5 Conclusions

The particle size of whey protein aggregates had a significant influence on the droplet size of O/W emulsions. Reduction in the droplet size achieved in this study by higher homogenisation conditions and protein concentration helped to achieve storage stability of 21 days for the emulsions. The use of whey protein aggregates conferred desirable rheological properties to the emulsions when compared to non-heated WPI. The emulsions turned into non-Newtonian fluids when whey protein aggregates were used instead of non-heated WPI. Thermal stability of emulsions stabilised by whey protein aggregates was affected by size of the aggregates. While all emulsions remained fluid on thermal treatment, droplet aggregation was noticed in the emulsions stabilised by non-heated WPI and whey proteins aggregates with higher particle size (133 nm). Emulsions stabilised by whey protein aggregates with smaller particle size of 40 – 55 nm did not flocculate further. The presence of whey protein aggregates at the interface and in the bulk phase appeared to increase the droplet aggregation as repulsive forces were reduced by reduction in pH and increase in ionic strength (addition of $CaCl_2$). While this could be negative for stability of fluid emulsions, it has several important implications in the formation of emulsion gels with high protein and reduced fat content. The desirable rheological properties achieved by whey protein aggregates could open the opportunities for development of dairy beverages and acidified milk beverages with high protein and reduced fat content. The future work on this subject could be conducted to investigate the role of oil content on the characteristic of emulsion. The emulsion gels could be formed by whey protein aggregates of varying particle size.

Chapter 5. Effect of oil content on characteristics of O/W emulsion stabilised by soluble whey protein aggregates

5.1 Abstract

Soluble whey protein aggregates are colloidal particles which can be formed by thermal treatment of whey protein solution at pH sufficiently away from the isoelectric point of whey protein and at low ionic strength. Soluble whey protein aggregates not only possess sufficient emulsifying property but also can act as effective rheology modifiers in O/W emulsions. A stable solution of soluble whey protein aggregates with a particle size of approx. 40 nm was prepared by heat treatment of whey protein isolate (WPI) solution at high WPI concentration of 8% w/w and 85 °C for 25 min. The WPI solution was maintained at pH = 6.9 prior to thermal treatment. It has been observed in a previous study that O/W emulsions prepared by using soluble whey protein aggregates in a similar range of particle size display a desirable shear thinning property in emulsions. In the present experimental work, the combined effect of soluble whey protein aggregates and change in oil content (10% to 30 % w/w) on the physical characteristics of O/W emulsions was studied. The physical characteristics of O/W emulsions measured as droplet size, ζ potential and apparent viscosity were significantly affected by both particle size and oil content of emulsions. The emulsions were also studied for their storage stability (up to 30 days) and thermal treatment (85 °C up to 25 min). For O/W emulsions stabilised by non-heated WPI, the emulsions remained as Newtonian fluids up to oil content of 25% w/w. The emulsions displayed a minor amount of non-Newtonian characteristic (measured using flow consistency index and flow behaviour index) at an oil content of 30% w/w. O/W emulsions stabilised by soluble whey protein aggregates with oil content < 25% displayed superior storage stability as well as thermal treatment stability.

5.2 Introduction

Whey proteins have been used as an emulsifier in O/W emulsions as well as food formulations (Dalgleish & Leaver, 1991; Euston & Hirst, 1999; Monahan et al., 1997; Singh et al., 2014). The rheology of O/W emulsions is significantly influenced by oil or fat content (Brighenti et al., 2008; Hussain et al., 2017). The food emulsions characterised by varying oil content, such as 10 – 15% (coffee creamer) (Golde & Schmidt, 2005), 20 – 40% (toppings, heavy cream, half and half cream, whipping cream) (Richardson et al., 1993), and high oil content of 65 – 80% (mayonnaise) (Ma & Boye, 2013), have different rheological properties and flow behaviours from Newtonian fluids to non-Newtonian fluids or viscoelastic semi-solids (D.J. McClements, 2015).

Fat or oil content plays an influential role in imparting desirable rheological, textural and sensory properties to an emulsion (D.J. McClements, 2015). Oil droplets in emulsion also influence other biological responses to the food in terms of hunger and satiety (Lett et al., 2016). Consumer buying preferences for food emulsions ae influenced by sensory properties such as mouthfeel, creaminess, and smoothness (Chung et al., 2015; Costell et al., 2010; Golding et al., 2011; Kindleysides et al., 2017). The perception of “creaminess” has emerged as the most important sensory aspect associated with fat content (Dickinson, 2018; Frøst & Janhøj, 2007; Mela, 1988). “Creaminess” has been shown to be closely linked to “thickness” and “smoothness” (Daget et al., 1987; Kokini & Cussler, 1983). For emulsions, viscosity at the shear rate of 50 s^{-1} has been widely used as it provides a sufficient measure of perceived “thickness” or “fat content” by a consumer (Kokini & Cussler, 1983; Kokini et al., 1977; Richardson et al., 1993)

The consumer demand for high protein and reduced fat products has been a significant increase in recent times to combat lifestyle ailments like obesity, hypertension, high blood sugar and diabetes (McIntosh et al., 1998; Smithers, 2008; Smithers, 2015). A rational design of reduced/low fat products would involve strategies to achieve viscosity of a full fat product to provide similar perception of “creaminess”. In the context of designing high protein and reduced fat emulsions, one could either use excess non-adsorbed protein to thicken the emulsion or use thickening agent or rheology modifier. It has been found in a previous study on emulsions stabilised by sodium caseinate that the rheology of emulsion can be modified by depletion flocculation induced by excess protein concentration in the emulsion (Dickinson

& Golding, 1997b). In a study with similar emulsions, it was observed that the stability of emulsions against creaming can be improved by using higher values of non-adsorbed proteins which led to stronger depletion flocculation and restricted the movement of oil droplets (Dickinson & Golding, 1997a). Unlike casein, it has been shown that the emulsions containing whey proteins do not undergo depletion flocculation even at higher protein concentration (> 4% w/w) (Dybowska, 2003; Euston & Hirst, 1999) because of its smaller size of 2-7 nm (Dalgleish & Leaver, 1991) when compared to colloidal particles of casein (20-50 nm) (Lucey et al., 2000). There have been attempts in the recent past to use ultra-high-pressure homogenisation to achieve desired rheological properties at low oil concentration (< 30% w/w) (Cortés-Muñoz et al., 2009; Hebishy et al., 2017; Soleimanpour et al., 2013). However, these studies noted that shear thinning behaviour was observed only at the oil content of > 30% in spite of higher homogenisation pressure.

A rather popular strategy to increase viscosity of emulsions at a low oil content has been use of thickeners, rheology modifiers or gelling agents in the formulation (Dickinson, 2009; McClements, 2000). Polysaccharides (hydrocolloids) like xanthan gum, gum arabic, pectin, sucrose diesters and starches as thickeners have been used in the past to increase the viscosity of emulsions (Akhtar et al., 2005; Ibanoglu, 2002; Mela, 1988; Mela et al., 1994; Sun & Gunasekaran, 2009) are used in several protein stabilised O/W emulsions for their ability to bind and immobilise water molecules or the bulk phase (Dickinson, 2003, 2009; Dickinson & Galazka, 1991; McClements, 2000). Besides thickening the emulsion, polysaccharides also provide longer term creaming stability to the emulsion even at lower oil content (Chung et al., 2013; Sun et al., 2007) by viscosity enhancement and depletion flocculation (McClements, 2000; Parker et al., 1995). Either of these mechanisms improves stability by immobilisation of oil droplets.

In recent times, there has been a steady rise in a consumer preference towards clean label (Asioli et al., 2017; Ozturk & McClements, 2016) which has prompted food manufacturers and food technologists to look for natural ingredients which do not bear an E number (Famelart et al., 2015; McClements et al., 2017; Setiowati et al., 2020) as they are considered to be a Generally Recognized As Safe (GRAS) status (de Castro et al., 2017). Soluble whey protein aggregates of varying physical characteristics, such as particle size, surface hydrophobicity, surface viscosity, intrinsic viscosity, and emulsifying capacity, can be prepared by controlled thermal denaturation and aggregation at pH ≫ pI and negligible ionic conditions (Moro et al., 2013; Moro et al., 2001; Schmitt et al., 2007). A few recent studies have been undertaken at low to moderate protein concentration (1 – 4% w/w) to assess the physical properties and kinetic stability of O/W emulsions stabilised by whey protein aggregates. However, most of these studies have been conducted at a constant oil content ranging from 30% (Chevallier et al., 2016; Dybowska & Krupa-Kozak, 2020) to 60% (Knudsen et al., 2008) and with a droplet size of emulsion close to 1 µm. A few these studies have assessed the impact of thermal treatment (60 to 95°C) on emulsions with a constant oil content of 30% (w/v) (Dybowska, 2011; Dybowska & Krupa-Kozak, 2020). However, these emulsions were prepared with aggregates formed at much lower concentration of 3% w/v of whey protein. The characteristics of emulsions with higher content of protein aggregates (> 6% w/w) and lower droplet size also have not been studied extensively. An earlier study reported by Boutin et al. (2007) evaluated the rheology of O/W emulsions and emulsion gels at varying oil content (10-20% of butteroil) and high protein concentration (8% w/w of whey protein aggregates) but did not evaluate storage and thermal stability of the emulsions. The study also noted that for a constant protein to oil ratio, viscosity (flow consistency index) of emulsions increased as homogenisation pressure was raised from 300 bar to 1200 bar. The O/W emulsions stabilised by high concentration of soluble protein aggregates would lead to a varying amount of non-adsorbed protein aggregates based on the oil content. It has been shown in the previous studies that intrinsic viscosity of protein aggregates (Vardhanabhuti & Foegeding, 1999) and viscosity of emulsions (Boutin et al., 2007) depend upon the concentration of aggregates (both at the interface and in the bulk phase).

The objective of this study was to evaluate the main characteristics (particle size, zeta potential, viscosity) of O/W emulsions prepared with varying oil content (10 – 30% w/w) with the use of non-heated (native) whey protein and soluble whey protein aggregates. Stability of emulsions was evaluated during storage for 21 days under controlled condition after thermal treatment. The outcome of the study would help in improving our understanding of soluble whey protein aggregates as a "fat replacer" which should enable its use in high protein reduced fat food formulations such as sauces, whipping creams and other dairy products.

5.3 Materials and Methods

5.3.1 Materials

WPI (Surestart WPI 895) containing 93.54% protein obtained from Fonterra Co-operative Ltd. (Auckland, New Zealand) was used as described in the previous chapters. Melrose MCT Pro-rapid oil which is coconut derived medium chain triglycerides was used as an oil phase which was obtained from Melrose Health, Australia. All other chemicals used in the experiment were of analytical grade. De-ionised water was used during the experiment.

5.3.2 Preparation of soluble whey protein aggregates

A stock solution of WPI (20% w/w) containing sodium azide (0.025 w/w) as antimicrobial agent was prepared as described in **Chapter 4.3.2** and then a WPI solution (8% w/w) was prepared. The WPI solution (8% w/w) was adjusted to pH 6.9 and stored at 4°C until used.

To prepare soluble whey protein aggregates, a 500 ml of whey protein solution was transferred into a Schott glass bottle with a membrane screw cap (Schott AG, Germany) and heated in a water bath at 85°C for 25 min. After heating, the solution containing whey protein aggregates was quickly cooled under tap water and kept in an ice bath for 20 min and then stored at 4°C for 24 hr before its use.

5.3.3 Preparation of O/W emulsions

The WPI solution containing soluble aggregates warmed at 20°C for 2 hrs was mixed with Melrose MCT Pro-rapid oil at ratios of 9:1, 8.5:1.5, 8:2, 7.5:2.5 and 7:3 (w/w). The mixtures were then homogenized to prepare a coarse O/W emulsion using a high shear mixer (Silverson L5, Silverson, UK) at 8,000 rpm for 5 min. To prepare a fine emulsion, the coarse emulsion was passed five times through a two-stage high-pressure homogeniser (APV 2000, SPXFLOW, Denmark) at the first and second pressures of 500 bar and 50 bar, respectively. The emulsions with the same concentrations of oil and whey protein were also prepared using non-heated WPI solution (pH 6.9) as the control samples. After preparation, the O/W emulsions were stored at 4°C until analysis. The whey protein aggregate dispersions as well as the O/W emulsions were prepared at least in duplicate in this study. The content of oil and whey protein in the emulsion samples and the sample codes designated with letter and number symbols for emulsions are shown in **Table 5.1**

Table 5.1 List of emulsion samples prepared and their oil and protein contents

Sample code	Oil content (% w/w)	WPI (% w/w)	Eff. protein conc. (% w/w)*	Temp (°C)	Heating time (min)
WA – 10	10	8	6.7	85	25
WA – 15	15	8	6.4	85	25
WA – 20	20	8	6.0	85	25
WA – 25	25	8	5.6	85	25
WA – 30	30	8	5.2	85	25
WPI – 10	10	8	6.7	Non - heated	
WPI – 15	15	8	6.4		
WPI – 20	20	8	6.0		
WPI – 25	25	8	5.6		
WPI – 30	30	8	5.2		

** Effective protein concentration which is the actual whey protein concentration calculated based on the WPI containing 93.54% and varying amount of continuous phase.*

5.3.4 Determination of oil droplet size of O/W emulsions

The particle size of oil droplets in emulsions was measured by dynamic light scattering (DLS) technique using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, England) as described in **Chapter 4.3.4**. The emulsion samples were diluted at a ratio of 1:500 respectively with de-ionised water prior to analysis. The droplet size of emulsions was presented as Z-average (based on intensity distribution).

5.3.5 Determination of ζ-potential of O/W emulsions

The ζ-potential of emulsion samples was measured as described in **Chapter 4.3.5**. The emulsion samples were diluted to a ratio of 1:1000 with de-ionised water before measurement to avoid multiple light scattering.

5.3.6 Determination of storage stability of O/W emulsions

A 25 ml aliquot of each O/W emulsion was taken and stored at 4°C for 1, 7, 15 and 30 days. The particle size and ζ-potential of the emulsions were measured during storage using Malvern Zetasizer Nano ZS in order to determine the storage stability of emulsions by monitoring the change of droplet size and ζ-potential.

5.3.7 Rheology of O/W emulsions

The viscosity of O/W emulsions was measured at 4°C by using a rheometer (AR 550, TA instruments) with a parallel plate geometry of 40 mm diameter as described in **Chapter 4.3.7**.

5.3.8 Effect of thermal treatment of O/W emulsions

The emulsion samples were heat treated at 85°C for 10, 20 and 25 min as described in **Chapter 4.3.10** to determine the stability of emulsions against thermal treatment by measuring the particle size of emulsion oil droplets.

5.3.9 Statistical Analysis

The preparation and analysis of samples were conducted at least in duplicate. The results are presented as mean values ± standard deviation. A one-way analysis of variance (ANOVA) was carried to determine the significance of treatment effect at a significance level ($P \leq 0.05$).

5.4 Results and Discussion

5.4.1 Droplet size and ζ-potential of O/W emulsions

The emulsions prepared using whey protein aggregates appeared to be rather viscoelastic in nature when compared to the emulsions prepared using non-heated WPI which were thinner (**Figure 5.1**). The oil droplet size and ζ-potential of all emulsions were measured 1 day after preparation. The results are shown in **Figure 5.2**. For the emulsions stabilised by whey protein aggregates, the droplet size was significantly affected by oil content ($P \leq 0.05$) with a significant increase with increasing oil content. The results could be expected as the emulsion containing higher oil concentration had lesser whey protein as shown in **Table 5.1**. The increase in oil droplet size was also observed in the emulsions prepared using non-heated whey protein but their particle size was much smaller than the emulsions prepared with whey protein aggregates, indicating the formation of oil droplets is bigger when whey protein aggregates are used as an emulsifier. It was also found that the change in oil droplet size with increasing oil content was less significant when non-heated WPI was used as an emulsifier.

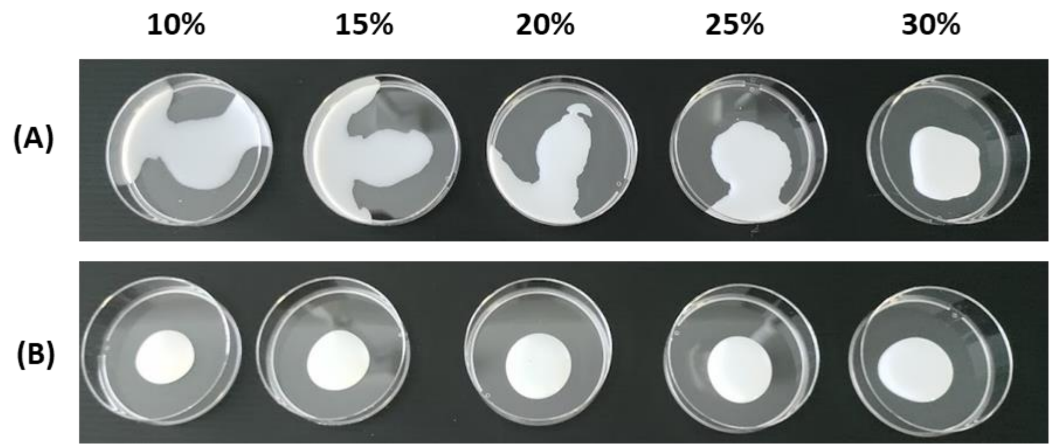


Figure 5.1 Visual appearance of O/W emulsions containing different oil concentrations (10, 15, 20, 25 and 30%) which were stabilised by **(A)** non-heated WPI and **(B)** whey protein aggregates

For the emulsions prepared with non-heated WPI and whey protein aggregates, ζ-potential was also significantly affected by oil content ($P \leq 0.05$) showing a gradual decrease in the ζ-potential value with increasing oil content (**Figure 5.2**). However, the values obtained were greater than -30 mV for all emulsions, indicating the presence of a reasonable electrostatic repulsive force acting on the oil droplets to provide kinetic stability to the emulsions. For a constant oil content, ζ-potential values obtained for the emulsions stabilised by non-heated WPI were greater than those obtained for the emulsions stabilised by whey protein aggregates. This confirms well with the earlier study (Chapter 4).

5.4.2 Storage stability of O/W emulsions

O/W emulsions are prone to destabilisation by a number of mechanisms, such as creaming, coalescence, flocculation, Ostwald ripening and gravitational sedimentation. Ostwald ripening can be safely ignored for all emulsions as MCT oil used in this study has poor solubility in water. Kinetic stability of all emulsions was evaluated on the basis of visual observations and the measurement of changes in droplet size during storage at 4°C. The storage study was conducted for 30 days, and droplet size was measured on Day 1,7, 15 and 30 (**Figure 5.3**). Based on the visual observation, all emulsions seemed stable during storage as they did not show any visual signs of creaming or phase separation. In addition, there was no phase separation or creaming detected on centrifuging the emulsions at 3000 g for 15 min as shown in (data not shown).

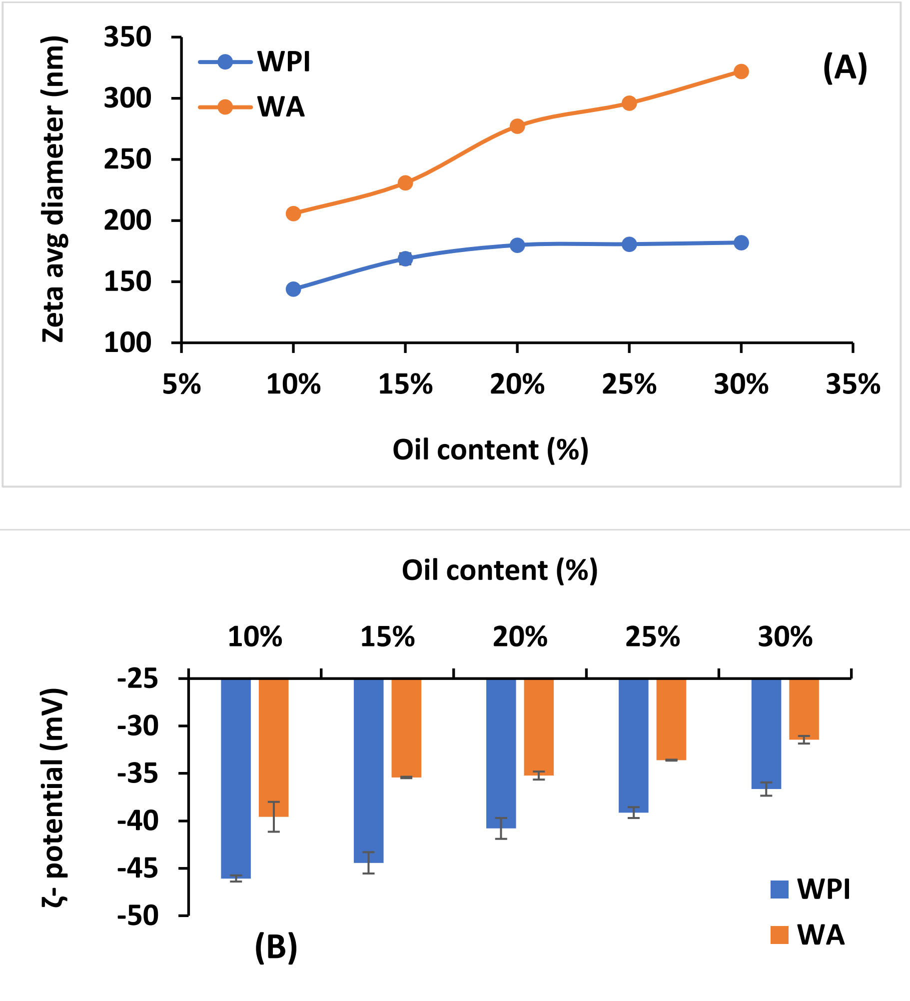


Figure 5.2 Particle size (A) and zeta potential (B) of O/W emulsions prepared using non-heated **WPI** and whey protein aggregates **(WA)**

However, for both emulsions stabilised by non-heated WPI and whey protein aggregates, the droplet size increased gradually during storage (**Figure 5.3**). Although it is not sure, besides the aforementioned emulsion destabilisation mechanisms, the increase in the droplet size during storage may also be attributed to a gradual accumulation of non-adsorbed proteins in the bulk phase on to interfacial layer (Dickinson & Matsumura, 1994).

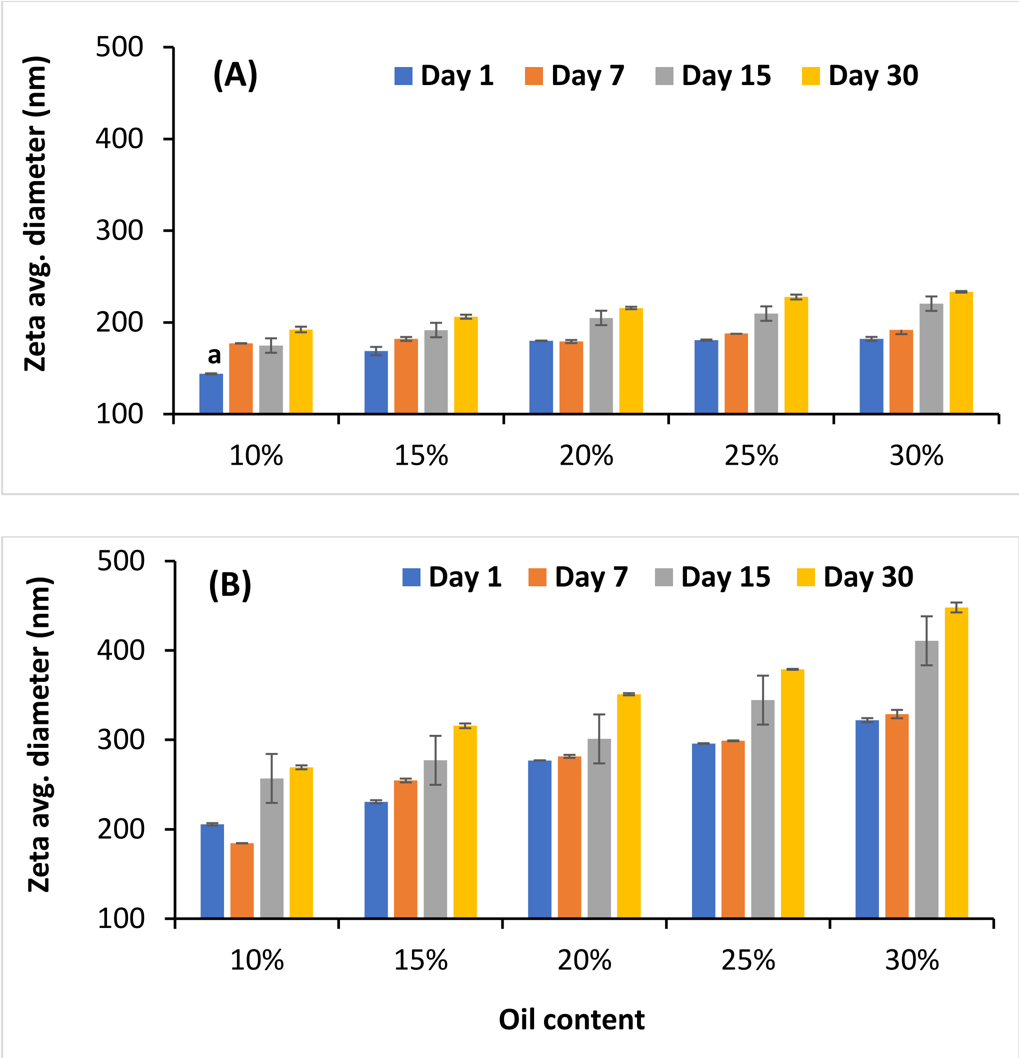


Figure 5.3 Storage stability of O/W emulsions stabilised by **(A)** non–heated **WPI** and **(B)** whey protein aggregates (**WA**) which was determined by measuring changes in particle size of oil droplets during storage at 4°C for 30 days.

As already mentioned above, the present study with increasing oil concentration significantly reduced the amount of protein available for coverage of droplets, thus resulting in a larger oil droplet size formed with increasing oil concentration. It has been reported that creaming stability of emulsions stabilised by non-heated whey protein is affected by surface load or protein concentration (Euston & Hirst, 1999). While the emulsion did not show any visible signs of phase separation or creaming, it could have undergone some of the destabilisation mechanisms (e.g. coalescence, flocculation) during storage which can be noticed from the bimodal particle size distribution of oil droplets, especially after storage for 30 days from the emulsions containing the highest oil content (30%) as shown in **Figure 5.4**.

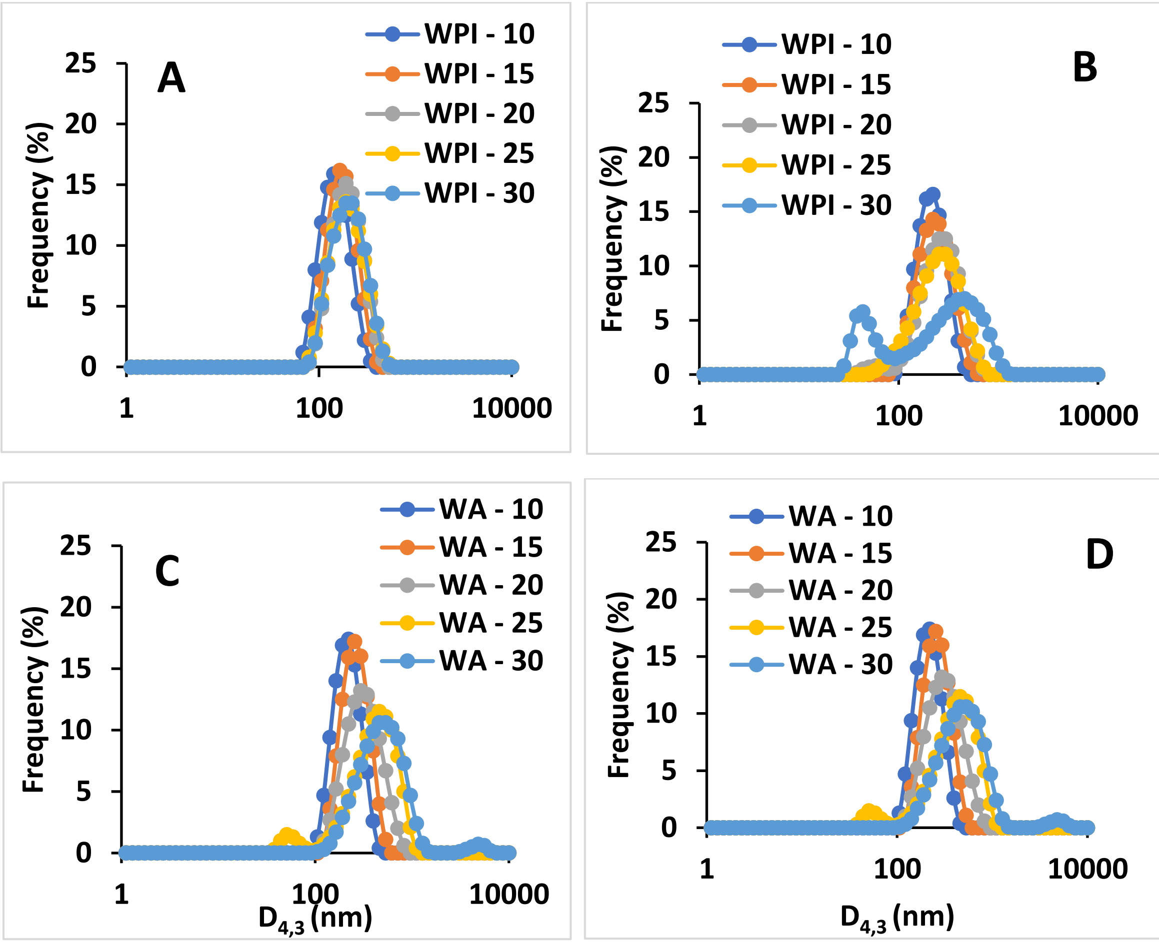


Figure 5.4 Changes in particle size distributions of O/W emulsions with different oil concentrations (10, 12, 20, 25, and 30%) stabilised by non-heated **WPI (A and B)** and **whey protein aggregates (WA) (C and D)** during storage at 4°C for **Day 1 (A and B)** and **Day 30 (B and D)**.

5.4.3 Rheology of O/W emulsions

The results of viscosity measurement from all emulsion samples are shown in **Table 5.2 and Figure 5.5**. The O/W emulsions stabilised by unheated whey protein behaved as a Newtonian fluid for oil content ≤ 25%. However, the emulsion containing 30% oil content displayed a weak shear thinning behaviour (K << 1) and (n<1) on account of increase in oil content. For non-flocculated emulsions with substantial repulsive forces, the viscosity of emulsions is directly proportional to the volume of dispersed phase (or oil content) (Bullard et al., 2009; Tadros, 2010). The emulsion has been observed to exhibit a shear thinning behaviour at oil content ≥ 30% (w/w) by some previous studies on whey proteins (Dybowska, 2011; Floury et al., 2000; Knudsen et al., 2008). The transition to shear thinning behaviour in the emulsion containing 30% oil stabilised by unheated WPI can be on account of "close-packing" of droplets resulting from a higher number of oil droplets (Tadros, 2010). For concentrated emulsions with oil content (ϕ) = 0.2 to 0.5, the viscosity of O/W emulsions can be expressed by Dougherty-Krieger (DK) equation (Equation 1) (Bullard et al., 2009; Roullet et al., 2019)

$$\eta = \eta_0\left(1 - \phi_{eff}/\phi_p\right)^{-1.75} \quad (1)$$

where, η = viscosity of the emulsion, η_0 = viscosity of continuous phase, ϕ_{eff} = droplet/aggregate volume fraction, and ϕ_p = maximum packing fraction (approx. 0.6 to 0.7).

Table 5.2 Rheology parameters measured at 20°C based on power law equation for all O/W emulsions.

Emulsion code	Rheology parameters			
	Viscosity[a] (mPa-s)	K (Pa-s^n)[b]	n[c]	R^2
WPI – 10	2.1 ± 0.0	0.002 ± 0.0001[a]	1	0.9961
WPI – 15	2.5 ± 0.0	0.0024 ±0.0002[a]	1	0.999
WPI – 20	4.2 ± 0.1	0.004 ± 0.0003[b]	1	0.998
WPI – 25	4.5 ± 0.2	0.0039 ± 0.0005[b]	1	0.998
WPI – 30	10.7 ± 0.2	0.0135 ± 0.004[c]	0.94 ± 0.01	0.994
WA – 10	51.2 ± 5.5	0.296 ± 0.04[d]	0.715 ± 0.04	0.993
WA – 15	110.8 ± 20.6	0.220 ± 0.03[d]	0.8 ± 0.015	0.974
WA – 20	153.1 ± 9.7	0.498 ± 0.05[e]	0.705 ± 0.01	0.986
WA – 25	721.1 ± 3.2	6.341 ± 0.09[f]	0.472 ± 0.02	0.972
WA – 30	1308.5 ± 18.5	18.24 ± 0.29[g]	0.325 ± 0.06	0.968

a) shear rate = 50 s^{-1}, b) Flow consistency index, c) flow behaviour index. Letters a,b,c,d,e,f and g mean statistically different ($P \leq 0.05$).

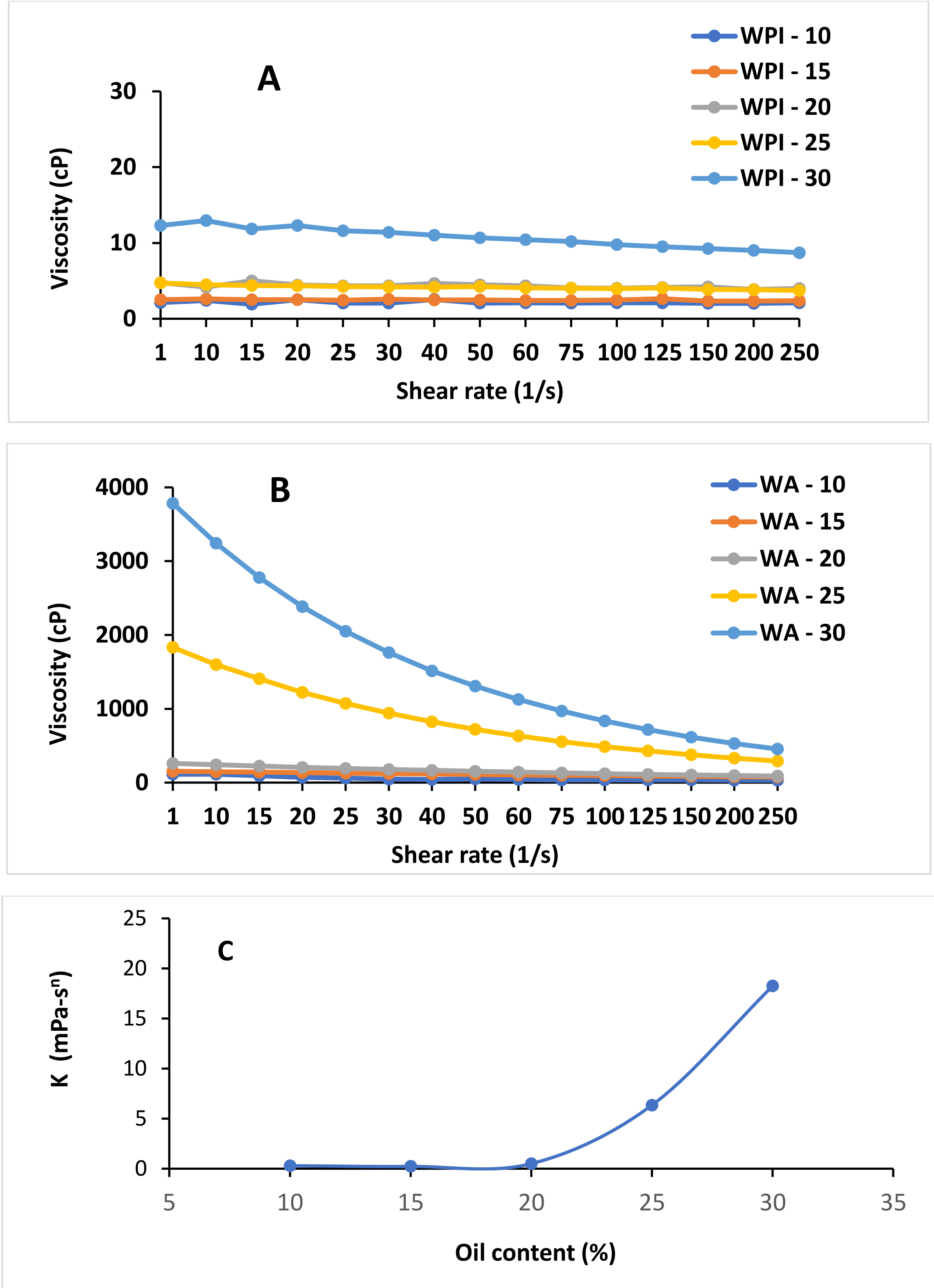


Figure 5.5 Viscosity of O/W emulsions measured at 20°C. **(A)** emulsions stabilised by non-heated **WPI**, **(B)** emulsions stabilised by whey protein aggregates **(WA),** and **(C)** flow consistency index of emulsions stabilised by whey protein aggregates

It was noted in Chapter 4 that the apparent viscosity of emulsion was significantly affected by particle size of whey proteins ($P \leq 0.05$) due to heating of whey proteins prior to the preparation of emulsions. The experimental values of apparent viscosity for O/W emulsions stabilised by unheated whey proteins and whey protein aggregates obtained in the present study (**Table 5.2**) confirms well with the results shown in Chapter 4. The higher values apparent viscosity observed for O/W emulsions could be due to increase in the viscosity of continuous phase (η_0) as indicated by rheology studies on dispersions of heated and non-heated whey proteins (Vardhanabhuti & Foegeding, 1999). For the O/W emulsions stabilised by non-heated whey proteins, ϕ_{eff} can be assumed to be equal to theoretical oil fraction ϕ as whey proteins form much thinner interfacial layer and would not materially increase the volume fraction. Although non-heated whey proteins would be present in continuous phase as excess non-adsorbed proteins, they do not contribute to increase in the viscosity of continuous phase. An increase in apparent viscosity of O/W emulsion stabilised by non-heated WPI and 30% oil was primarily due to higher volume fraction of droplets with increase in oil content.

For the O/W emulsions stabilised by whey protein aggregates, the apparent viscosity of emulsions was significantly affected by oil content ($P \leq 0.05$). All emulsions exhibited a shear thinning behaviour with $n < 1$. The value of "n" decreased with increase in oil content (**Table 5.2 and Figure 5.5B**). The flow consistency index (K) increased significantly with oil content ($P \leq 0.05$) (**Figure 5.5C**). In an earlier study on emulsions stabilised by whey protein aggregates by Boutin et al. (2007), shear thinning behaviour was observed in emulsions with oil content of $\geq 10\%$ with a similar trend in the values of flow consistency index.

Based on the work by Quemada (1977) and Berli et al. (2002), an amended form (Equation 2) of Equation 1 can be used to model rheology of flocculated emulsions.

$$\eta = \eta_0 \left(1 - \phi_{eff}/\phi_m\right)^{-2} \quad (2)$$

where, ϕ_{eff} = volume fraction of flocs, ϕ_m = maximum packing fraction (0.79)

Whey protein aggregates in the continuous phase behave as colloidal particles. Depletion flocculation is observed in emulsions stabilised by whey protein aggregates due to large amount of non-adsorbed whey proteins (Boutin et al., 2007). It has been shown in earlier studies on whey protein aggregates (Boutin et al., 2007; Dybowska, 2011; Dybowska & Krupa-Kozak, 2020) and sodium caseinate (Dickinson & Golding, 1997a) that excess concentration of such colloidal particles leads to depletion flocculation and such fluids exhibit a shear thinning behaviour. The present study consists of emulsions with high WPI content (8% w/w; actual whey protein content from 5.2-6.7%). For emulsions with oil content $\leq$ 20%, there would be a significant amount of non-adsorbed whey protein aggregates in the continuous phase. The steady increase in viscosity as the oil content was increased from 10% to 20% indicates a larger contribution of non-adsorbed whey protein aggregates (Boutin et al., 2007; Euston & Hirst, 1999). The presence of non-adsorbed whey protein aggregates leads to the higher viscosity of continuous phase. The droplet aggregation due to depletion flocculation leads to higher values of ϕ_{eff} compared to theoretical oil content (Tadros, 2010).

A sharp increase in the apparent viscosity for oil content > 20% is due to combined effect of droplet aggregation due to depletion flocculation as well as equally significant increase in droplet volume fraction on account of higher oil content. In a recent study on sodium caseinate (Roullet et al., 2019), it has been noted that ϕ_{eff} = 2.16 ϕ. For oil content 25% and 30%, this would lead to values of ϕ_{eff} = 0.55 and 0.65. At such higher values, droplets are closely packed with each other and a subsequent rapid rise in the apparent viscosity.

5.4.4 Effect of thermal treatment of O/W emulsions

Emulsions stabilised by non-heated WPI were prepared with high concentration of WPI (8% w/w). However, the amount of non-adsorbed protein would be lower with increase in the oil content. Also, on account of smaller droplet size of unheated emulsions (refer to Figure 5.3) achieved through higher homogenisation conditions, the interfacial area would increase with increase in oil content (Hebishy et al., 2017; Karleskind et al., 1996). Hence, it could be assumed that with increase in oil content, there would be lower amount of non-adsorbed protein in the bulk phase (Dickinson, 1992b, 1994). On thermal heat treatment, all emulsions remained fluid and did not show any visible creaming. The particle size measurement was conducted one day after thermal treatment. The results of mean particle size and particle size distribution of emulsions are shown in **Table 5.3** and **Figures 5.6-5.7**, respectively.

All emulsions displayed a gradual increase in the particle size of oil droplets which was expressed as Zeta avg. diameter. An increase in droplet size is believed to be due to thickening of interfacial layer as disulphide bonds formed due to protein-protein interaction at interfacial layer (Damodaran & Anand, 1997; Monahan et al., 1996). There have also been previous studies which have shown the formation of disulphide bonds and non-covalent bonds between denatured proteins (due to thermal heating) in the bulk phase and interfacial layer (Fang & Dalgleish, 1997; Tcholakova et al., 2006). For the oil content < 20%, it can be assumed that there was non-adsorbed proteins as indicated by an earlier studied on WPI (Euston & Hirst, 1999). The study indicated that surface coverage of non-heated WPI reaches plateau at around 4% w/w. Although homogenisation pressure of 200 bar used in that study was much lower than this study, it can be considered that there was a significant amount of non-adsorbed proteins available in WPI -10% and WPI – 15% emulsion samples. Based on visual

Table 5.3 Effect of thermal treatment (85^{o}C for 10, 20 and 25 min) on O/W emulsions stabilised by whey protein aggregates and non-heated WPI.

Type of Emulsion	Zeta -avg diameter (nm)			
	Unheated	85 °C /10 min	85 °C /20 min	85 °C/ 25 min
WPI – 10	177.1 ± 0.0	189.7 ± 4.5	201.4 ± 8.7	213.5 ± 0.0
WPI – 15	181.9 ± 4.2	193.1 ± 3.8	207.4 ± 2.5	211.0 ± 0.5
WPI – 20	179.1 ± 6.8	189.8 ± 5.5	210.0 ± 9.4	238.6 ± 9.8
WPI – 25	188.0 ± 4.2	189.3 ± 0.1	197.5 ± 1.7	218.0 ± 4.6
WPI – 30	191.8 ± 2.1	205.6 ± 3.2	212.8 ± 0.8	216.0 ± 0.8
WA – 10	184.4 ± 0.0	205.0 ± 1.2	212.4 ± 0.7	218.0 ± 4.6
WA – 15	254.6 ± 2.1	266.1 ± 1.2	272.7 ± 2.6	276.6 ± 0.0
WA – 20	281.1 ± 1.7	301.6 ± 4.2	317.5 ± 0.6	323.8 ± 3.0
WA – 25	299.0 ± 0.4	315.6 ± 2.0	381.6 ± 1.9	431.5 ± 2.1
WA – 30	322.0 ± 2.3	368.3 ± 7.1	385.6 ± 5.3	478.9 ± 2.1

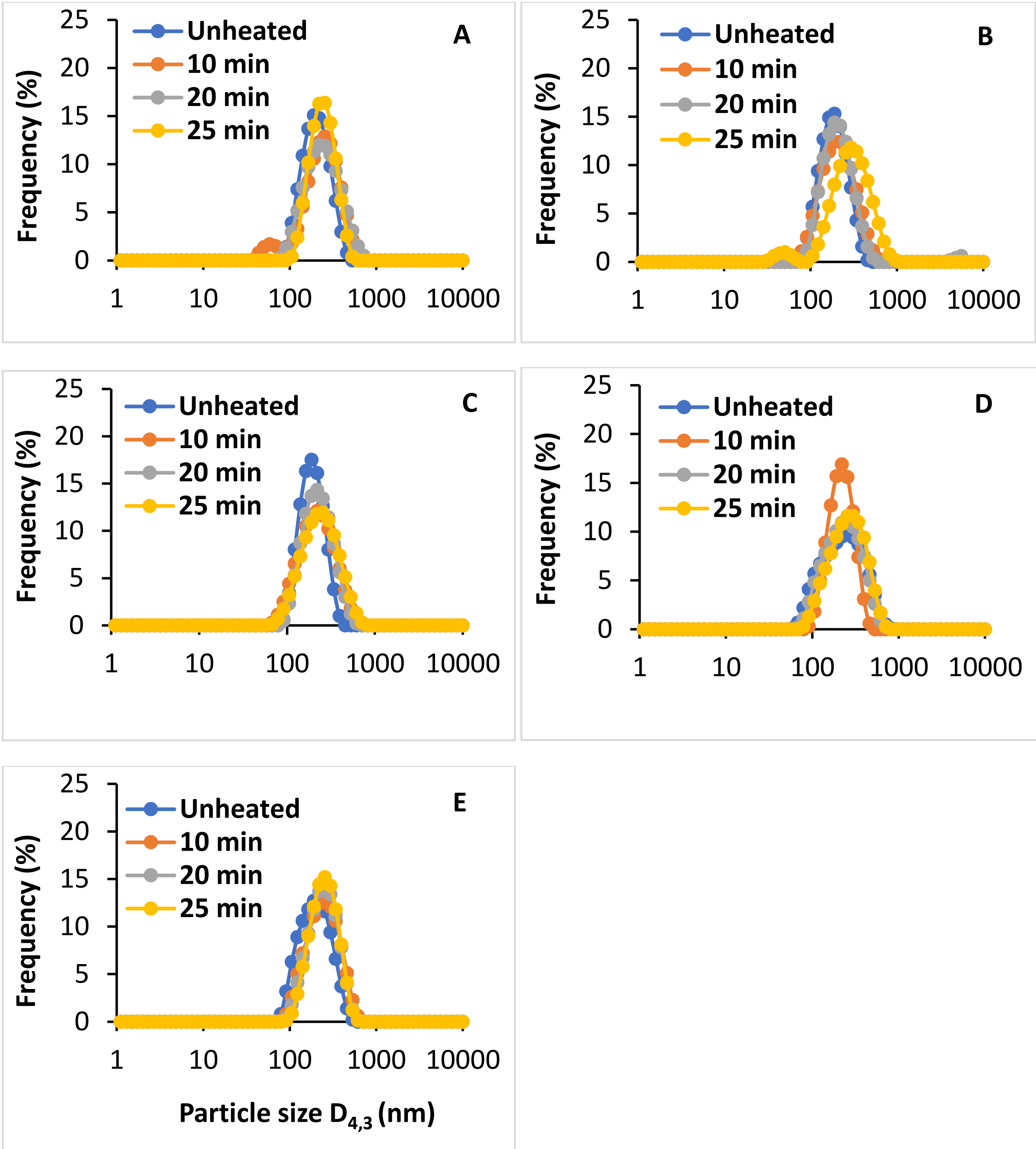


Fig 5.6 Particle size distributions of oil droplets in emulsions stabilised by **non-heated whey protein** which were heat treated at 85C for 10, 20 and 25 min or without heat treatment. **(A)** WPI 10% oil, **(B)** WPI 15% oil, **(C)** WPI 20% oil, **(D)** WPI 25% oil, and **(E)** WPI 30% oil.

observation, all emulsions appeared to be stable with no visual evidence of creaming and phase separation due to heat treatment. All emulsions stabilised by whey protein aggregates also remained fluid after thermal treatment of 85°C for 10-25 min. This confirms well with the observations noted in the earlier study (Chapter 4) with an oil content of 15% and same concentration of protein aggregates. There was a gradual increase in the droplet size for WA 10, WA 15 and WA 20 (**Table 5.3**). Although at an oil content of 10% to 20% one would expect substantial quantity of whey protein aggregates, it has been shown in previous studies that beverages containing whey protein aggregates (concentration = 5 to 7% w/w) had an improved thermal stability compared to non-heated whey proteins due to higher overall negative charge and surface hydrophobicity (Ryan & Foegeding, 2015).

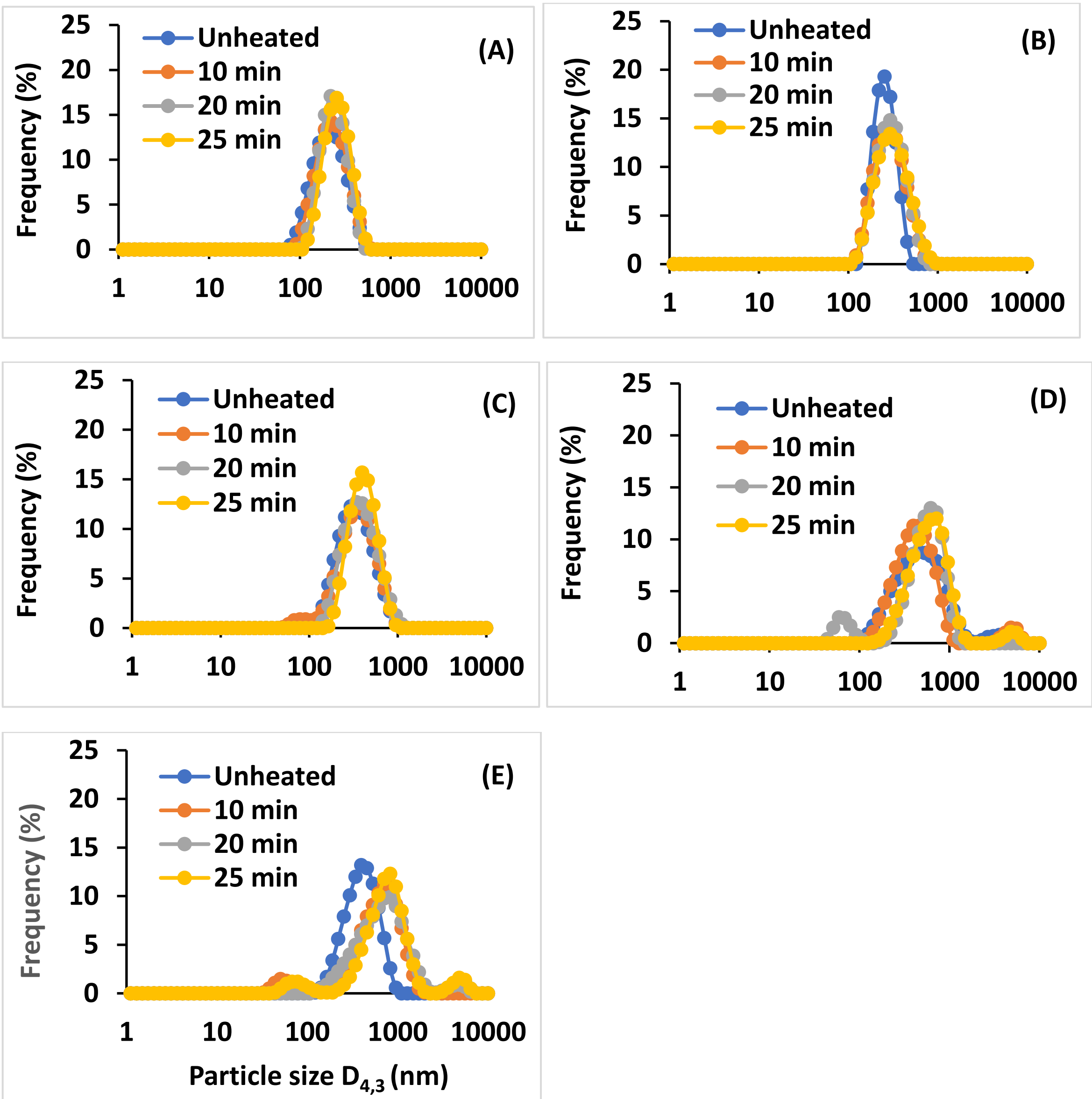


Fig 5.7 Particle size distributions of oil droplets in emulsions stabilised by w**hey protein aggregated (WA)** which were heat treated at 85C for 10, 20 and 25 min or without heat treatment. **(A)** WA 10% oil, **(B)** WA 15% oil, **(C)** WA 20% oil, **(D)** WA 25% oil, and **(E)** WA 30% oil.

For emulsions stabilised by whey protein aggregates, it has been observed in an earlier studies that thermal stability O/W emulsions increased with the presence of whey protein aggregates at the interface and the continuous phase (Dybowska & Krupa-Kozak, 2020). The study compared the stability of O/W emulsions stabilised by non-heated WPC and whey protein aggregates (3% w/w) for 30% oil content on thermal treatment at 60 to 95°C for 5 min. The study noted that while the emulsions stabilised by whey protein aggregates remained flocculated for all heat treatments at different temperatures used, they remained fluid. Higher thermal stability was attributed to preference for protein aggregation over droplet aggregation. An earlier study on thermal treatment of emulsions stabilised by whey protein concentrate with a significant amount of non-adsorbed proteins (Euston et al., 2000) concluded that the aggregates formed by thermal treatment could destabilise the emulsion. Destabilisation could occur if droplet aggregation is favoured over protein aggregation. An earlier study on impact of sucrose on thermal stability of O/W emulsions stabilised by whey proteins concluded that flocculation did not occur in spite of a higher amount of non-adsorbed protein aggregates in the bulk phase (Kulmyrzaev et al., 2000). The increase in the concentration of sucrose facilitated protein-protein interaction or aggregation over droplet aggregation. However, the mechanism or the reasons for such a preference remains unexplained.

In this study, for the effect of heat treatment (85ºC for 10-25 min) on whey protein aggregates-stabilised emulsions, the results of particle size distribution measured from the emulsions with and without heat treatment are shown in **Figure 5.7**. For the emulsions WA 25% and WA 30%, their particle size distributions appeared to be bimodal when the duration of heat treatment applied was 20 min or 25 min. There was a significant increase of 44% and 48%, respectively, in the droplet size of emulsions at the end of 25 min of heating at 85°C. However, there was no creaming or phase separation observed from these emulsions. This could be due to the droplet sizes which were still small being less than 500 nm in all case. In addition, the viscosity of some emulsions with higher oil concentration might have restricted the movement of oil droplets, thus preventing from significant droplet aggregation, coalescence and creaming.

In a previous research reported on retort treatment of O/W emulsions stabilised by 4% WPI and varying amount of xanthan gum (0.1% to 1%) (Ye & Singh, 2006), it was shown that the emulsions did not cream at xanthan gum concentration of 0.2% and 0.4% on account higher viscosity of the emulsion due to the formation of network structure (Parker et al., 1995).

5.5 Conclusions

The physical characteristics of O/W emulsions measured as droplet size, ζ-potential and apparent viscosity were significantly affected by both particle size and oil content of emulsions. For non-Newtonian emulsions stabilised whey protein aggregates with constant particle size, apparent viscosity of emulsions was significantly affected by oil content. The O/W emulsions behaved as semi-solids for an oil content $\geq$ 25%. A modification in rheological behaviour on account of use of soluble whey protein aggregates confirms that such whey protein particles can be effectively used as "fat replacers" or rheology modifiers to prepare high protein and clean label dairy products. On thermal treatment, all emulsions remained stable to phase separation. However, the proportion of non-adsorbed whey protein particles in O/W emulsions prior to thermal treatment could have a significant impact of droplet distribution. A presence of excess whey protein particles in the continuous phase could lead to droplet flocculation due to depletion interaction although this was not able to be measured accurately. Overall, the O/W emulsions containing 10-30% oil prepared by using unheated whey protein or whey protein aggregates as emulsifiers were stable during storage for 30 days. Also, the emulsions remained stable against thermal treatment as there was creaming or phase separation detected although there was a gradual increase in the particle size of oil droplets during storage and by thermal treatment with increasing heating time. Future work could involve characterisation of emulsions using microscopy to gain better understanding of the interface and structure of whey protein aggregates in the bulk phase.

Chapter 6. Overall Conclusions and Future Recommendations

The broad goal of this research project was to investigate the possibility of using a form of whey protein to enhance its functionality as a functional ingredient. The experimental work was undertaken to improve our understanding of soluble whey protein aggregates and its use as an emulsifier and as a rheology modifier. An equally important aim of this endeavour was to build a toolkit for formulation of high protein and low fat O/W emulsions and emulsion gels. However, emphasis of this experimental work was to investigate use of such soluble whey protein aggregates in O/W emulsions. With this in mind, a higher concentration of WPI at 8% w/w was selected for the formation of soluble whey protein aggregates and further use in O/W emulsions. This would confirm to 21 CFR guidelines for label claim of "high protein" in subsequent products. Soluble whey protein aggregates of varying particle size were successfully synthesised by using a varying combination of heating temperatures (68, 75 and 85°C), duration of heating (up to 60 min.) and pH (6.9, 6.4 and 6.0). A stable colloidal dispersion (ζ- potential strength higher than -35 mV) of soluble whey protein aggregates in a size range of 40 - 130 nm was successfully synthesized during the experimental work. An emphasis was laid on yield of such soluble whey protein aggregates to allow for its further use in real world applications. A yield of more than 80% could be achieved at a heating temperature of 68 and 85°C for all levels of pH (6.9, 6.4 and 6.0) used in this study. A rather significant deviation in yield was observed when heating a WPI solution with pH 6.0 at 75°C. A significant drop in yield to 34.5% was observed at this process condition. While such an increase in the formation of large insoluble aggregates has been noted in earlier studies due to a combination of decrease in denaturation rate of β-lactoglobulin and enhanced involvement of denatured α–lactalbumin, the underlying mechanism is an unknown. Further studies could focus on the measurement of denaturation rate of WPI as well as the characterisation of aggregates formed at these process conditions. This could help us identify the reasons for such anomalous behaviour.

O/W emulsions were prepared by soluble whey protein aggregates to investigate the ability of such particles to function as an emulsifier as well as a rheology modifier. The present study found that the O/W emulsions stabilised by soluble whey protein aggregates possess desirable rheological properties compared to those stabilised by non-heated WPI. The study also found that O/W emulsions ranging from a fluid to a semi-solid state can be prepared by changing the concentration of soluble whey protein aggregates and oil. Future studies could focus on comparing the rheological properties of O/W emulsions stabilised by soluble whey protein aggregates with similar emulsions stabilised by polysaccharides.

The use of soluble whey protein aggregates improves the stability of emulsions to thermal treatment. However, the stability is found to diminish when oil content is $\geq$ 25%. Such emulsions display an increase in droplet aggregation. While no visible phase separation was observed, kinetic stability of such emulsions subjected to thermal treatment should be conducted in future. Thermal treatment of 85°C up to 25 min may not be sufficient in real world scenario. Future studies could focus on assessing thermal stability of O/W emulsions stabilised by whey protein aggregates for higher heating temperature (> 120°C like UHT).

Storage stability of O/W emulsions measured by noting droplet aggregation was found to be much superior in case of those stabilised by whey protein aggregates compared to non-heated WPI. However, O/W emulsions stabilised by whey protein aggregates undergo significant droplet aggregation at pH 4 and 5 and $CaCl_2$ concentration of $\geq$ 5mM. While it destabilises fluid emulsions, it could be effectively used to develop emulsion gels with a range of textural and mechanical properties. Future studies could look at encapsulating bioactives in such O/W emulsions and emulsion gels. These could be used as desirable food textures for preparing nutraceuticals for ageing adults. It could be even more desirable for ageing adults with diminished dental health. The use of soluble whey protein aggregates could materially expand in the arena of high protein nutraceutical beverages if gastro-intestinal studies could be combined with clinical studies to understand the efficacy of such whey protein particles in enhancing the delivery and assimilation of bioactives in the human body.

Note: The present experimental work was hampered by frequent lockdowns in Auckland on account of Covid 19 pandemic. During the period of experimental work, Auckland underwent a cumulative lockdown period of more than 200 days with one of the lockdown extending to more than 100 days. Under normal circumstances, the research work could have been performed better. It affected my original plan to undertake a few more research work to supplement existing experimental work.

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