Physico-chemical and functional properties of whey proteins as affected by the source of whey

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Dedicated to my beloved parents,

Indika, Methuki & Miheli

Abstract

Increasing global demand for fermented dairy products and salted hard cheese varieties has led to the vast generation of acid and salty whey waste streams. Unlike sweet whey, acid and salty whey are not conveniently processed to whey protein concentrates (WPC) or isolates (WPI) due to compositional variations mainly characterized by elevated lactic acid and salt concentrations, respectively. Therefore, the necessity of developing a suitable processing method in converting acid and salty whey waste streams to usable concentrate powders is importantly emphasized by the dairy industry. Factors affecting the behavior of the whey proteins (WPs) present in these two whey streams, during or post-production are unknown. Hence, the present study was proposed to establish the physico-chemical behavior of the WPs present in acid and salty whey and their behavior during application of traditional concentration and spray drying. To gain an insight into the applicability of these WPC powders in commercial applications as food ingredients, current study further proposed evaluating their molecular, physical and functional property changes during storage. The proposed outcomes were achieved in five separate phases.

During the first phase, the physico-chemical characteristics of WPs in sweet, salty and acid whey were investigated and compared with those of native whey, which underwent minimum commercial processing. Acid whey used in the current study was generated during Greek yoghurt manufacturing. Four whey streams were analyzed for the composition, interactional properties and molecular structures. WPs from acid whey were characterized by hydrophobically and covalently driven protein aggregation. Covalent aggregation in acid whey consisted of both thiol/disulphide and non-thiol/disulphide mediated reactions. Fourier transform infrared (FTIR) data characterized this protein aggregation as β-sheet attractions

causing subtle changes in the secondary structure. In contrast, WPs in salty whey aggregated via van der Waals, hydrogen, electrostatic interactions and covalent bonds. Both thiol/disulphide and non-thiol/disulphide interactions led to cross-linked β-sheets, disrupting the secondary protein structures. This aggregation exposed hydrophobic segments while oxidizing a high number of free thiol groups. The absence of these types of WP aggregation in sweet or native whey highlighted the fact that elevated salt concentration in salty whey or heat treatment applied during production of acid whey are largely responsible for structural differences.

In second phase, the influence of physico-chemical characteristics of liquid-WPCs obtained by ultrafiltration of acid and salty whey streams on the surface composition, particle organization, secondary structures and protein interactions of the respective spray dried WPC powders was investigated. Their properties were compared with those of native and sweet whey. Acid whey concentrate demonstrated characteristically low surface charge, high surface hydrophobicity, high average particle size and high thiol activity compared with sweet and native whey concentrates. Salty whey concentrate was characterized by low surface hydrophobicity, high thiol activity and low average particle size. Surface characterization of WPC powders revealed protein-rich surfaces for all whey powders while those in salty whey were highly hydrophobic. Protein characteristics of native and sweet whey WPC powders largely followed those of concentrates. In contrast, protein characteristics of the acid and salty whey WPC powders largely changed from those of the liquid WPCs.

In third phase, changes of the secondary structure and protein interactions of WPs present in native, sweet, acid and salty-WPC powders were analyzed following storage at 4,

25 or 45 °C and 22 or 33% relative humidity (RH) for a period of 90-days. WPs aggregated predominantly through covalent crosslinking, achieving maximum at 45°C and 33% RH. Greater participation of β-lactoglobulin (β-LG) in covalent crosslinking was evident in all WPC powders, while that of α-lactalbumin (α-LA) was significantly ($p < 0.05$) high in acid-WPC powder only. Reaction order of β-LG denaturation in acid and salty-WPCs was approximately 2, while approximately 1 in native and sweet-WPC powders. Activation energy was significantly ($p < 0.05$) higher in native and sweet-WPC powders, with averages recorded as 97 and 49.8 $kJmol^{-1}$, respectively, than that in acid and salty-WPC powders with averages of 27.5 and 33.8 $kJmol^{-1}$, respectively, mainly attributed to the inherently high concentrations of lactic acid (LA) and salts in these WPC powders.

Storage conditions may compromise stability of WPs in dry state, which is also influenced by their inherent composition. Thus, in fourth phase, physical characteristics of native, sweet, acid and salty-WPC powders were analyzed during storage at several temperatures (4, 25, 45°C) and RH levels of 22 and 33% for a period of 90 days. Particle surface of native, sweet and acid-WPC powders was dominated by proteins under all storage conditions, while fat and minerals prevailed on the surface of salty-WPC powder. Compared to native- and sweet-WPC powders, origin of acid- and salty-WPC powders influenced these streams to be rich in minerals, primarily accumulated in the particle core. Hydrophilic nature of the core impacted redistribution of proteins within the particle during storage. Compared to native- and sweet-WPC powders, particles were cohesively arranged in acid- and salty-WPC powders, which in turn changed physical properties such as particle size and surface charge. Elevated storage temperatures induced protein denaturation, melting of surface free

fat and lactose crystallization in WPC powders, while humidity regulated the molecular mobility in these reactions.

In addition to the influence on physical characteristics, combined impact of storage conditions and composition of WPC powders may affect the functional characteristics of WPs thus affect their functionality. Therefore, in the fifth phase, functional characteristics of native, sweet, acid and salty-WPC powders were analyzed after storage at 25°C and RH levels of 22 and 33% for 90 days. Native-, sweet- and acid-WPC powders exhibited a high solubility (97–82%), which was largely retained during storage. In contrast, the solubility of salty-WPC started at ~52-55% and gradually increased by ~5% during storage. Ionic sodium in salty-WPC interlinked WPs through salt bridges and charge screening, exposing reactive sites for intensive aggregation. Heat stability of salty-WPC was the highest (64s), while lowest was recorded for native-WPC (16s). In the presence of ionic sodium in salty-WPC, WPs denature due to ionic-bridging, charge screening and osmotic effects leading to intensive aggregation. High emulsion activity was recorded for salty-WPC powder, while those for other WPC powders were similar. Emulsion stability varied as native-> acid-> salty-> sweet-WPC. High number of hydrophobic segments was likely exposed on the protein surface in salty-WPC powders due to sodium-induced WP denaturation, thus increasing the number of proteins absorbed to the emulsion interface, enhancing the emulsion activity. Functionality of different WPC powders predominantly depended on the inherited composition and storage conditions.

In conclusion, the inherent composition of acid and salty whey streams affects physico-chemical properties of WPs present in these whey streams, their liquid WPCs and spray dried WPC powders. Furthermore, properties of WPs present in these WPC powders

were influenced by the compositional differences, processing effects of these two whey streams and storage conditions, which can be observed at molecular level, in turn affecting their physical characteristics. Furthermore, compositional differences showed a combinational impact with storage conditions on the functional properties of acid and salty WPC powders. Therefore, the unique composition of acid and salty whey is a factor that must be manipulated strategically in order to achieve a commercially viable production process and storable WPC powders.

CERTIFICATE

Prof. Todor Vasiljevic, PhD (University of Alberta)

Professor of Food Science

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This is to certify that the thesis entitled "**PHYSICO-CHEMICAL AND FUNCTIONAL PROPERTIES OF WHEY PROTEINS AS AFFECTED BY THE SOURCE OF WHEY**" submitted by **Manjula Nishanthi Kottahachchi Kankanamge** in partial fulfillment of the requirement for the award of the Doctor of Philosophy with specialization in Food Sciences and Technology at Victoria University is a record of bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Professor Todor Vasiljevic

(Principal supervisor)

Date: 03.05.2018

DECLARATION

I, Manjula Nishanthi Kottahachchi Kankanamge, declare that the PhD thesis by Publication entitled **"PHYSICO-CHEMICAL AND FUNCTIONAL PROPERTIES OF WHEY PROTEINS AS AFFECTED BY THE SOURCE OF WHEY"** is no more than 100, 000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes.

This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Manjula Nishanthi Kottahachchi Kankanamge

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Date: 04.05.2018

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PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

Item/ Chapter No.	Paper Title	Publication Status (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	Publication Title and Details (e.g. date published, impact factor etc.)
3	Properties of whey proteins obtained from different whey streams	Published	International Dairy Journal: SJR Q1
4	Compositional and structural properties of whey proteins of sweet, acid and salty whey concentrates and their respective spray dried powders	Published	International Dairy Journal: SJR Q1
5	Properties of whey protein concentrate powders obtained by spray drying of sweet, salty and acid whey under varying storage conditions	Published	Journal of Food Engineering: SJR Q1
6	Physical properties of selected spray dried whey protein concentrate powders during storage	Published	Journal of Food Engineering: SJR Q1
7	Impact of storage conditions on solubility, heat stability and emulsifying properties of selected spray dried whey protein concentrates	Published	LWT - Food Science and Technology: SJR Q1

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Date:

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Awards, Oral presentation and poster presentations

Awards

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Oral presentations

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List of abbreviations

AA – Amino acid

Ala – Alanine

Asn – Asparagine

Asp – Aspartate

AWC – Acid whey concentrate

BSA – Bovine serum albumin

Ca – Calcium

$CaCl_2$ – Calcium chloride

CCP – Colloidal calcium phosphate

Cl – Chloride

Cys – Cysteine

DSC – Differential scanning calorimetry

DTNB – 5,5-Dithio-bis (2-nitrobenzoic Acid)

EAI – Emulsion activity index

ESI – Emulsion stability index

FTIR – Fourier transform infrared

GLM – General Linear Model

GMPs – Glycomacropeptides

Glu – Glutamate

Gly – Glycine

HCl – Hydrochloric acid

HCT – Heat coagulation time

HPLC – High performance liquid chromatography

ICP – Inductively coupled plasma

IgA – Immunoglobulin A

IgD – Immunoglobulin D

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IgM – Immunoglobulin M

Igs – Immunoglobulins

Ile – Isoleucine

K – Potassium

LF – Lactoferrin

LA – Lactic acid

Lys – Lysine

Leu – Leucine

$MgCl_2$ – Magnesium chloride

Mg – Magnesium

Mw – Molecular weight

Na – Sodium

NaCl – Sodium chloride

NCN – Non-casein nitrogen

NPN – Non-protein nitrogen

NWC – Native whey concentrate

PAGE – Polyacrylamide gel electrophoresis

Phe – Phenylalanine

RH – Relative humidity

RP-HPLC – Reversed phase high performance liquid chromatography

SDS^{R} – Reducing Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS^{NR} – Non-reducing Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM – Scanning electron microscope

StWC – Salty whey concentrate

SWC – Sweet whey concentrate

Thr – Threonine

TN – Total nitrogen

UF – Ultrafiltration

UV – Ultraviolet

Val – Valine

WPC – Whey protein concentrate

WPI – Whey protein isolate

WPs – Whey proteins

TCA – Trichloro acetic acid

XPS – X-ray Photoelectron Spectroscopy

β-LG – β-Lactoglobulin

α-LA – α-Lactalbumin

pK_a – Acid dissociation constant

n – Order of reaction

K_n – Rate constant

E_a – Activation energy

S_0 – Surface hydrophobicity

w/w – Weight per weight

Chapter 1: Introduction

1.1 Background

After coagulation of caseins during cheese and yoghurt manufacturing, the remaining water with its soluble components, which has been discharged in the past as a liquid waste, is generally termed as "whey". Whey comprises of ~50% of milk solids, including 20% of the milk proteins, lactose, fat and minerals. Whey can be mainly categorized as sweet whey and acid whey based on the method of casein precipitation applied in the production of different cheese varieties and acid coagulated products. For instance, the acid precipitation of caseins in manufacturing cottage or Ricotta cheese and strained yoghurt yields the acid whey as a by-product, whereas, sweet whey originates from the production of rennet type hard cheeses. Some sweet whey is sub-categorized as salty whey due to the higher salt concentrations applied during the salting step of some cheese varieties such as Cheddar and Colby.

Proteins in whey, commonly termed whey proteins (WPs), are recognised as an excellent nutritional and dietary supplement. Different whey powders are manufactured mainly from the liquid sweet whey as whey protein concentrate (WPC), whey protein isolates (WPI), demineralized whey powders, delactosed whey powder and individual WP fractions such as β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and lactoferrin. The major steps involved in manufacturing whey powders are concentration through membrane filtration, evaporation, lactose crystallization and spray drying. These powders are widely used as ingredients for various applications such as in energy supplements, infant formula, bakery products, desserts and confectionaries. Due to the increased production of cheese and yoghurt products, the volumes of generated acid and salty whey streams have increased, creating a need for their processing.

However, efforts to further processing of acid and salty whey into powders have had a limited success due to the inherent compositional differences of these streams in comparison to that of sweet whey.

Acid whey and salty whey are comparatively high in acidity and salinity, respectively, in comparison to sweet whey (Blaschek et al., 2007; Josephson, Rizvi, & Harper, 1975). Acid whey contains more lactic acid (LA) and calcium (Ca), while salty whey contains more sodium (Na) as compared to sweet whey. As a result of these compositional variations, WPs present in these whey streams are characterized with unique structural and interactional properties (Nishanthi, Vasiljevic, & Chandrapala, 2017). For instance, the high acidity in acid whey leads to aggregation of WPs via hydrophobic interactions and covalent linkages (Nishanthi et al., 2017). In contrast, WPs tend to aggregate via strong covalent bonds and weak van der Waals, hydrogen and electrostatic interactions in salty whey due to its high salinity (Nishanthi et al., 2017). LA lowers the charge repulsion between proteins allowing the protein molecules to approach closely, thus forming various inter-molecular interactions (Israelachvili, 2011). Ca and Na salts also interact with WPs through ionic-bridging and charge screening. Divalent cations such as calcium form ionic-bridges between the carboxyl groups of adjacent WPs, therefore crosslinking the proteins (Zhu & Damodaran, 1994). Cations have the capacity to screen the negative charge distribution on a protein surface, hence reducing the inter-molecular distance (Zhu & Damodaran, 1994). These interactions in turn open up the tertiary structure of WPs, therefore, exposing the reactive sites such as the hydrophobic sites located in the β-LG calyx and the free thiol group located in the H strand of β-LG for reactions (McSweeney, 2013). The presence of protein aggregates and the high concentrations of Ca and Na salts restricts the concentration steps through membrane filtration during the manufacturing of powders. Clogging of membrane pores by inorganic

salts and protein aggregates leads to extended filtration times, thus increasing the cost of production. In addition, the presence of LA in acid whey possesses problems such as improper crystallization of lactose leading to the formation of clumps that attaches to the dryer wall during drying. This in turn affects the powder yield.

In addition to processing difficulties, the storage conditions can induce changes in the protein conformation at a molecular level differently from one whey stream to another, thus affecting the functional properties of their respective powders. Intermolecular covalent and non-covalent interactions occur between WPs, resulting in protein unfolding and denaturation, eventually leading to reversible and irreversible aggregation. Norwood et al. (2016) showed that after 3 months storage of WPI powders at 40 ºC, a proportion of the native WPs decreases with a concomitant increase in proportion of denatured proteins. Such changes were not observed at 4 ºC or 20 ºC with up to 15 months of storage. At elevated storage temperatures, the flexibility and mobility of the WPs increases, resulting in the partial loss of their tertiary structures. Therefore, the surface properties of WPs change during storage, increasing the surface hydrophobicity after storage at 60 ºC and 22 % relative humidity (RH) for one month (Burgain et al., 2016). In addition to WP denaturation, lactosylation, crystallization of lactose and melting of fat occur during storage of WPC powders. Lactosylation forms brown colour pigments and affect the digestibility of the proteins. Conversion of amorphous lactose to crystalline lactose forms a network in the particle interior, leading to a phase separation between lactose and other components (Kim, Chen, & Pearce, 2009), releasing free fat onto the particle surface (Kim et al., 2009), subsequently causing particle agglomeration. The storage of WPC powders at high temperatures causes melting of the surface free fat, forming particle agglomerates, which in turn affect the solubility, wettability and flowability of the powders.

In the current literature, WP properties and associated changes have been mostly studied on WPCs produced from sweet whey. However, no research has been conducted on acid and salty whey and their respective WPC powders so far. The findings for sweet whey cannot be directly applied to acid and salty whey, predominantly due to their unique compositional differences. Therefore, the current study carries the significance of establishing the fundamental knowledge of physico-chemical properties of WPs in acid and salty whey liquid streams, their liquid WPCs obtained after membrane filtration and the spray dried powders. Furthermore, the current study applies this fundamental knowledge in understanding the changes that occur in molecular, physical and functional levels in WPC powders during storage. The knowledge generated in the current study about acid and salty could be considered in implementing novel techniques for further processing.

1.2 Specific objectives

The following specific objectives were initially set and consequently achieved:

1. Establishing the physico-chemical characteristics of WPs present in sweet, acid and salty whey in comparison with those of native whey that was not subjected to any commercial processing
2. Investigating the influence of compositional variations of different whey streams (native, sweet, acid and salty) in relation to the surface and structural properties of the WPs during concentration and spray drying
3. Ascertaining conformational and surface interactional changes of WPs present in sweet, acid and salty WPCs stored at three different temperatures (4, 25 and 45 °C) and two relative humidity (RH) levels (22% and 33%) over a period of 90 days

4. Exploring the physical changes of sweet-, acid- and salty-WPC powders subjected to several storage conditions including three different storage temperatures (4, 25 and 45 °C) and two RH levels (22 and 33%) over a storage period of 90 days.

5. Evaluating the functional characteristics of stored sweet-, acid- and salty-WPC powders at 25°C and two different RH levels (22 and 33%) for a period of 90 days

1.3 Thesis outline

Chapter 1 provides background, research objectives and the outline of the thesis. Chapter 2 presents a review of current scientific knowledge on different WPs, their structures, WP denaturation and aggregation, different whey streams, manufacturing of WPCs, technical drawbacks of processing of acid and salty whey, functional properties of WPC powders and physico-chemical changes of WPC powders during storage. Chapter 3 investigates the physico-chemical characteristics of WPs present in sweet, acid and salty whey. Chapter 4 presents a study on how compositional variations of native, sweet, acid and salty whey streams influences their surface and structural properties of WPs during concentration and spray drying. Chapter 5 reports the conformational and surface interactional changes of WPs present in sweet, acid and salty WPCs under three different storage temperatures (4, 25 and 45 °C) and two RH levels (22% and 33%) over a storage period of 90 days. Chapter 6 presents the findings on physical changes of sweet, acid and salty-WPC powders subjected to above storage conditions. Chapter 7 reports the functional characteristics of sweet, acid and salty-WPC powders subjected to storage at 25°C and two different RH levels (22 and 33%) stored for 90 days. Finally, conclusions of the overall study and future directions of the research are included in Chapter 8.

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Chapter 2: Literature review

2.1 Milk

Milk is a very complex mixture of nutrients, which has the primary function of nourishing the young. Bovine milk has become an essential food in the day to day diet of humans of all ages. Therefore, the demand for bovine milk and relevant products is continuously increasing globally. The average composition of bovine milk consists of 87% water, 3.3% protein, 4% milk fat, 4.6% lactose, 0.65% milk salts and 0.18% organic acid (Walstra, Wouters, & Geurts, 2005). Milk can be considered as an emulsion of fat-in-water, where fat globules are stabilized by the phospholipid and glycoprotein membrane in the aqueous phase (Figure 2.1).

2.1.1 Milk proteins

Milk proteins, which are one of the most important constituents in milk, are sub-categorized into two groups as "casein" and "whey proteins" based on their solubility at pH 4.6 (Huppertz, Fox, de Kruif, & Kelly, 2006). Caseins make up 80% of the total protein content, while the remaining 20% is whey proteins. Caseins are insoluble at pH 4.6 and present mostly as a colloidal particle named the "casein micelle" in the milk serum phase (Figure 2.1). Whey proteins are globular and are soluble at pH 4.6.

2.1.2 Casein and the casein micelle structure

Caseins are classified into four main proteins as α_{s1}-, α_{s2}-, β- and κ-caseins. Each protein consists of a single polypeptide chain, in which the amino acid sequence is different, and thus possesses different properties. The average molecular weight of caseins is in the range of ~20-24 kDa. All caseins are phosphorylated to various extents with binding of one phosphoseryl residue to κ-casein, 8 or 9 residues to α_{s1}-casein and 4 or 5 residues to β-casein. Calcium (Ca), the main mineral associated with the caseins, is bound mainly via the

phosphoseryl residues and the binding capacity varies as α_{s2}-casein > α_{s1}-casein > β-casein. Caseins have a high hydrophobicity.

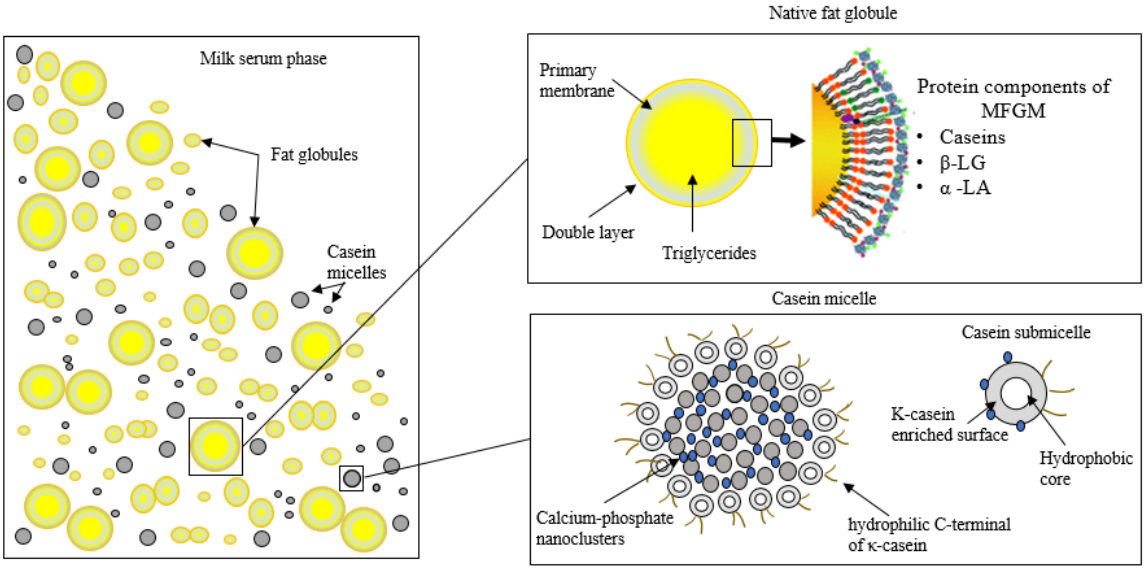

Figure 2.1 – Schematic representation of milk structure

At neutral pH and low ionic strength, α_{s1}-casein exists as a monomer, whereas increasing the ionic concentration initiates self-aggregation. Similar to α_{s1}-casein, α_{s2}-casein self-aggregates with the increase in ionic concentration, but self-aggregation is disturbed by the ionic strength above 0.2 M. The self-association of β- or κ-casein can be described as a monomer-polymer equilibrium due to their micellar-like amphiphilic behavior. Hydrophobic interactions between the C-terminal segments and the charged N-terminal segments lead the self-association among these proteins. Self-association of β-casein strongly depend on the temperature, ionic strength and pH, whereas, polymerization of κ-casein is independent of temperature and ionic strength.

Another important feature of caseins is the ability to form disulphide bonds. There are two cysteine residues in α_{s2}-casein and one in κ-casein (McSweeney & Fox, 2013). Therefore, α_{s2}-casein participates in both intramolecular and intermolecular disulphide bond formation,

while κ-casein only forms intermolecular disulphide bonds during polymerization (McSweeney & Fox, 2013).

Caseins are cross-linked mainly through hydrophobic interactions and colloidal calcium phosphate (CCP) nanoclusters within the casein micelle (Figure 2.1). Micelles contain ~ 92% (w/w) casein and 8% (w/w) inorganic salts, mainly present as calcium phosphate. The micelle structure is porous with an average diameter of 200 nm. The outer layer of the micelle is formed by equal molar amounts of α_s- and κ-caseins while the inside is mainly composed of β- and α_s-caseins and with a small amount of κ-casein (McSweeney, 2013). Several models have been proposed for the structure of the casein micelle, however, the structure is still a controversial area of dairy science (Horne, 2006). In common, the casein micelle is believed to be composed of several sub units, linked together through colloidal calcium phosphate. The hydrophilic C-terminal of κ-casein is located at the surface of the micelle and sticks out to provide a hairy layer. This hairy layer provides the electrostatic and steric stabilization to the micelle to be dispersed in the aqueous phase (De Kruif & Holt, 2003).

2.2 Whey proteins (WPs)

WPs are globular proteins with a uniform distribution of hydrophobic and hydrophilic amino acids. The six main types of WPs are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), immunoglobulins, lactoferrin and lactoperoxidase (Table 2.1).

Table 2.1: Composition of WPs in milk (Gupta & Prakash, 2017; McSweeney, 2013; Patel, 2007)

Whey protein	Percentage of total WPs (%)
β-Lactoglobulin	50.0
α-Lactalbumin	20.0
Bovine serum albumin	5.0
Lactoferrin	1.0
Immunoglobulins	10.0
Lactoperoxidase	0.25 – 0.5

2.2.1 β-Lactoglobulin (β-LG)

β-LG is the primary WP representing ~50% of the amount of total WPs (Oldfield, 1996). The isoelectric point of β-LG is around 5.2. The molecular weight of β-LG is about 18.3 kDa and consists of 162 amino acids. β-LG naturally occurs in a very compact globular structure with four β sheets forming a β barrel type structure that provides ideal sites for hydrophobic interactions (Croguennec, 2004). Further, the β-LG monomer contains five cysteine residues, out of which four are involved in forming two disulfide bonds and one exist as a free thiol group. Two disulphide bonds occur between Cys_{106} - Cys_{119} and Cys_{66} - Cys_{160}, while the free thiol group is situated at Cys_{121}. The two disulphide bonds and the free thiol group are buried inside the native β-LG structure (Figure 2.2).

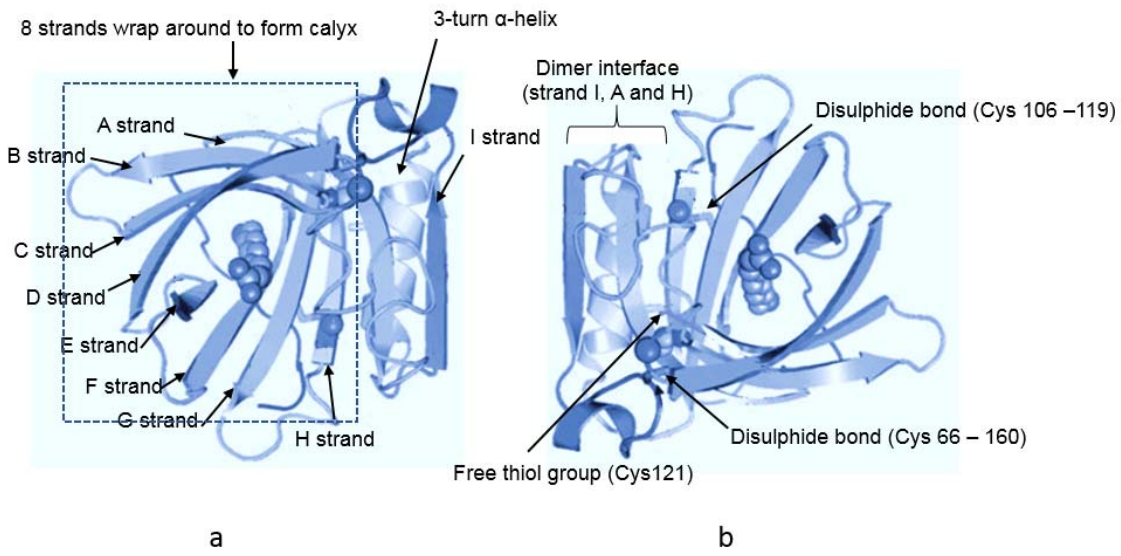

Figure 2.2: Diagram of dimeric structure of bovine β-LG A. (a) showing nine β-strands, 3-turn α-helix and the formation of calyx (Strands labelled A to H form the calyx). (b) showing two disulphide bonds, one free thiol group and the formation of dimer interface (Strand 1 forms the dimer interface with strand A and H). Sourced from Edwards, Creamer, and Jameson (2009)

Native β-LG consists of nine antiparallel β-strands. Eight of the β-strands are wrapped around to form a flattened, conical barrel called the "calyx". This calyx is closed at one end by Trp_{19} while strand A bends 90° around residues 21 – 22 forming an antiparallel interaction with strand H to complete the calyx. The calyx is cylindrical, while its walls are hydrophobic. Unless occupied by a ligand, the calyx remains empty. Strand I, which is the ninth strand, is located outside of the monomer and forms the dimer interface together with strand A and H. The dimer interface is buried 570 Å on each monomer, and involves antiparallel interactions of residues 146-150 with those of the other sub unit (Sakurai & Goto, 2002). A 3-turn α-helix is located over the strand H and on the outer surface of the calyx. The polypeptide chains between the β-strands forms two separate 3_{10}- turns and a γ-turn (McSweeney, 2013). The free thiol group is covered by the α-helix, however, depending on the denaturation conditions,

it is exposed to the outside, initiating a chain of reactions preceding with sulphydryl-disulphide interchange reactions. In a solution of β-LG, the approximate contents of α-helix, β-sheets and random coil are 8%, 45% and 47%, respectively (McSweeney, 2013).

Several genetic variants of β-LG have been reported with the A and B variants occurring at almost equal frequencies under most conditions. The main difference in the A and B variants is the presence of different amino acids at position 64 and 118, where Asp and Val are present in the β-LG A variant, respectively while those were replaced by Gly and Ala in the β-LG B variant (McSweeney, 2013). Variant A is more flexible than the variant B.

2.2.2 α-Lactalbumin (α-LA)

α-LA is the second major WP comprising ~20% of the total WP concentration. At neutral and alkaline pH conditions, α-LA exists as a nearly spherical, compact globular protein. Unlike β-LG, α-LA is mostly present as a monomer, consisting of 123 amino acid residues, with a Mw of 14 kDa. The structure of the α-LA molecule consists of three regular α-helices, two regions of 3_{10} helix and a three-stranded antiparallel β-sheet separated by irregular β-turns (Figure 2.3). The molecule has eight Cys residues, which are formed into four disulphide bonds as Cys_{6}-Cys_{120}, Cys_{28}-Cys_{111}, Cys_{60}-Cys_{77} and Cys_{73}-Cys_{90} (Figure 2.3). The disulphide bonds of Cys_{6}-Cys_{120} and Cys_{28}-Cys_{111} are located in the helical lobe, while the disulphide linking between Cys_{60}-Cys_{77} is located in the β-sheet containing lobe. The remaining disulphide bond is formed between Cys_{73} located in the β-lobe and Cys_{90} of the helical lobe. As all Cys residues have participated in forming disulphide bonds, α-LA does not have a reactive free thiol group. However, the disulphide bond between Cys_{6}-Cys_{120} is found to be more sensitive to cleavage than the other three, due to its lower inherent stability (Kuwajima, Ikeguchi, Sugawara, Hiraoka, & Sugai, 1990). Spectral analysis of α-LA in

solution suggests that α-LA contains 20% of α-helix, 14% of β-sheet and 60% of unordered structures (McSweeney, 2013).

α-LA can be categorized as “apo” and “holo”, in which the former is the Ca bound form. The holo form of α-LA is the most abundant form present in milk. Calcium binding stabilizes the conformation providing a high thermal stability. The specific Ca-binding site is located between the 3_{10} helix of the β-lobe and helix C of the α-lobe (McSweeney, 2013) (Figure 2.3). The Ca ion is coordinated by the oxygen atoms of the Asp_{82}, Asp_{87} and Asp_{88} side chain carboxyl groups and the peptide carbonyl oxygen of Lys_{79} and Asp_{84} (McSweeney, 2013). The amino acid sequence of this region is Lys_{79}-Thr_{80}-Leu_{81}-Asn_{82}-Asn_{83}-Asp_{84}-Leu_{85}-Thr_{86}-Asn_{87}-Asn_{88}. The expansion of this Ca-binding site due to charge repulsion between the negatively charged Asp residues, affects the orientation of the secondary structure elements, mainly 3_{10} helix and α-helix C (McSweeney, 2013). These structures are connected by a disulphide bond between Cys_{73}-Cys_{90}. In addition, these structural changes can lead to a separation between the α and β subdomains and disturb the hydrophobic interactions (McSweeney, 2013). This process is known as the initial step of the unfolding of α-LA that leads to the formation of a molten globule state.

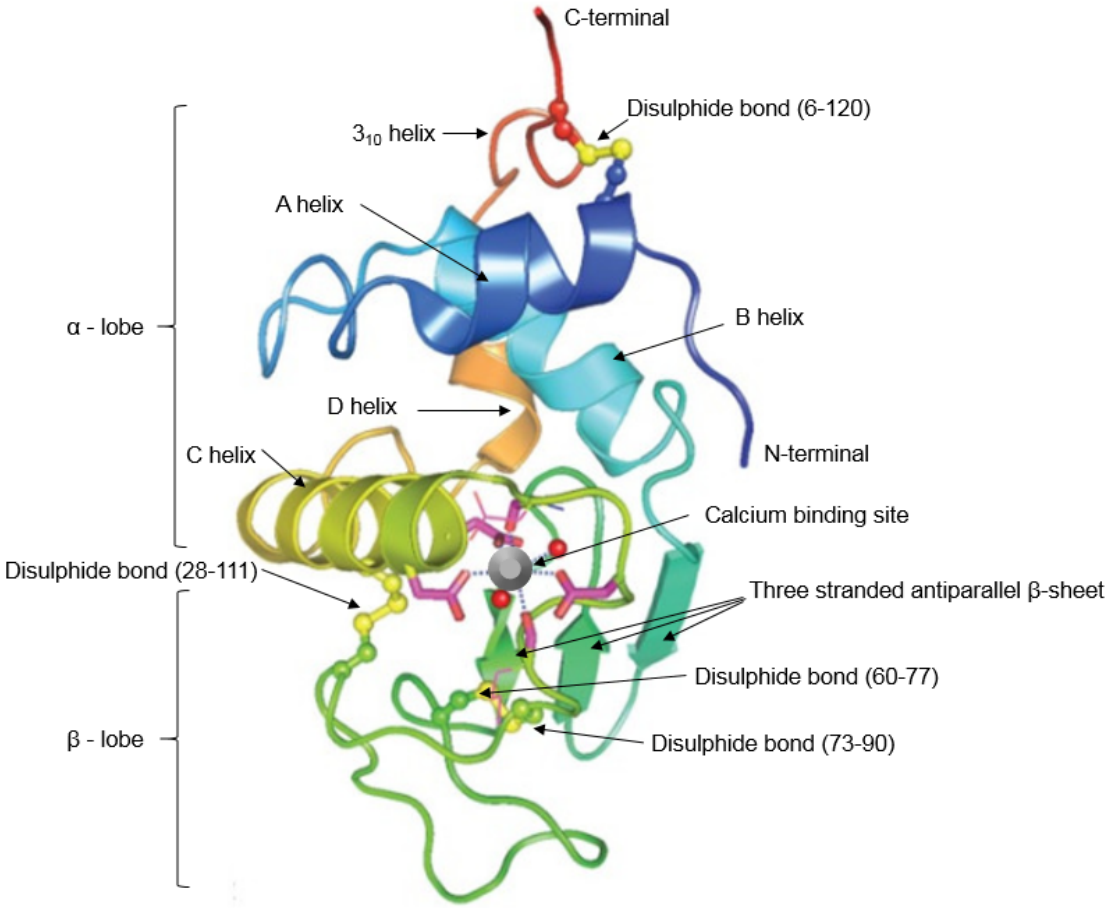

Figure 2.3: Schematic diagram of the bovine α-lactalbumin. Two lobes are labelled as α-lobe and β-lobe. Sourced from Boland, Singh, and Thompson (2014)

2.2.3 Bovine serum albumin (BSA)

The concentration of BSA in bovine milk alters with the stage of lactation, where the concentration increases during late lactation. BSA exists as a monomer and contains 583 amino acid residues, weighing up to 66 kDa (McSweeney, 2013). This molecule has nine loops stabilized by 17 intramolecular disulphide bonds. It also contains one free thiol group at position 34. BSA is characterized by an overall oblate shape with three domain structures, named I, II and III, each stabilized by an intramolecular network of disulphide bonds (McSweeney, 2013). Each domain consists of two large double loops and one small double

loop, showing different degrees of hydrophobicity and surface charge. Ligand binding is a prominent feature of BSA. BSA consists of ~5 – 12 binding sites under neutral pH conditions, allowing binding of several ligands, such as fatty acids and cations.

The secondary structure of BSA consists of ~66 % of helix, 10 % of β-turns and 23% of extended chains, with no β-sheets (McSweeney, 2013). The helix formation is limited due to the large number of disulphide bonds. However, these disulphide bonds are comparatively short, and provide a certain flexibility.

2.2.4 Lactoferrin

Lactoferrin (LF) is a single chain protein with 689 amino acids and a Mw of ~80 kDa (McSweeney & Fox, 2013). The molecular structure of LF contains 14 intramolecular disulphide bonds, with no reactive free thiol groups. LF has a high isoelectric point of pH 8.7 comparative to the other WPs. The polypeptide chain of LF has two lobes with similar amino acid sequences. The N-lobe consisted with amino acids 1-333 and the C-lobe with 345-692. Each lobe consists with two sub-domains as N1, N2 and C1, C2. The two lobes are connected by an extended α-helix (Figure 2.4). There are two binding sites in each lobe for iron and glycan. The conformations of the N-terminal lobe and the C-terminal lobe have different affinities for iron binding. The conformation of the iron -free, apo state is more open and flexible.

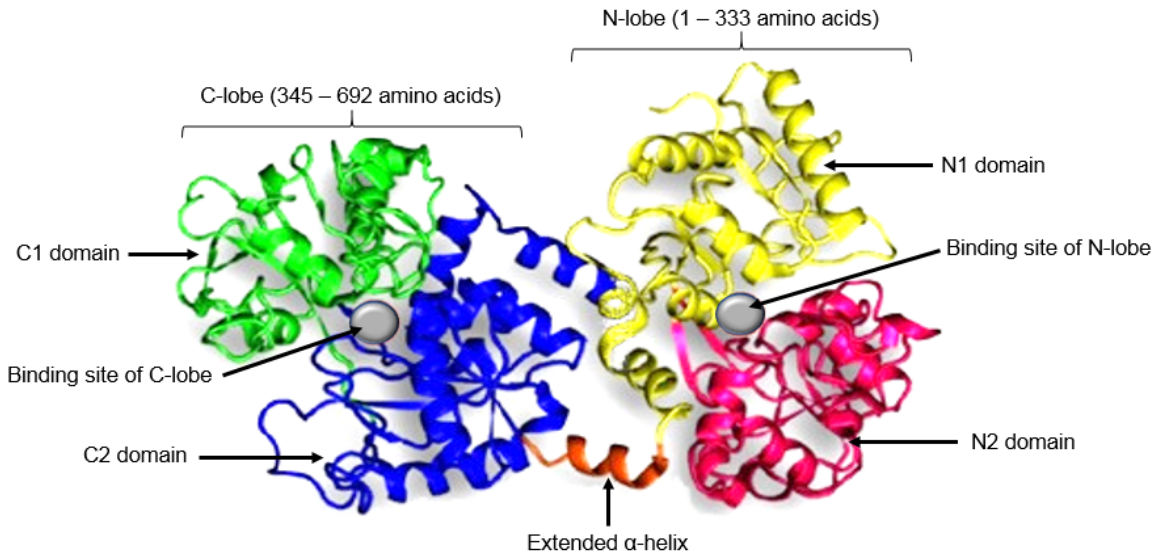

Figure 2.4: Schematic diagram of the bovine lactoferrin. Two lobes are labelled as N and C. N lobe (1 – 333 amino acids) is divided to N1 and N2 domains, colored in yellow and pink, respectively. C lobe (345 – 692) is divided to C1 and C2 domains, colored in green and blue, respectively. The extended α- helix interconnecting the lobes is colored in orange. Grey colored sphere represents the binding site of each lobe. Sourced from Sharma, Sinha, Kaushik, Kaur, and Singh (2013).

2.2.5 Immunoglobulins

Immunoglobulins (Igs) are a group of complex large glycoproteins that have antibody activity. These proteins represent approximately 10% of the WPs. Five distinct types of Igs occur in bovine milk: IgA, IgM, IgE, IgD and IgG (McSweeney, 2013). These Igs have a similar basic structure with the Mw varying between 150 – 900 kDa. Type IgG is subdivided into two groups as IgG_1 and IgG_2. IgG_1 is the main type in bovine milk, representing ~80% of the total Igs. Igs are present as monomers or polymers, consisting with four polypeptide chains. Out of those four polypeptide chains two chains are small with low Mw (~22 kDa) and two chains are bigger with high Mw (~50 – 70 kDa) (Patel, 2007). Two small polypeptide chains differ in their amino acid chain structure, but are similar in their amino acid sequence

(Oldfield, 1996). The light chains and heavy chains are connected by a disulphide bond, while the heavy chains are held together by another disulphide bonds (McSweeney, 2013).

2.2.6 Lactoperoxidase

Lactoperoxidase is a type of milk enzyme accounting for 0.25–0.5 % of the total WPs (Table 2.1). It is the most abundant enzyme in whey following the curding process. It has the property to catalyze certain reactions, and to reduce of hydrogen peroxide, which can inhibit a range of bacterial species. This enzyme is heat tolerable up to ~75 °C, but heating at 78°C for 15 s completely destroy its activity (Griffiths, 1986). Therefore, lactoperoxidase rapidly inactivated during the pasteurization process (Griffiths, 1986).

Overview of properties of Igs and lactoperoxidase can be found in other references (Bogahawaththa, Chandrapala, & Vasiljevic, 2017; De Wit & Van Hooydonk, 1996). For the objectives of this thesis, these were not considered to be major proteins, and thus were not covered in depth.

2.3 Whey streams from dairy manufacture

The remaining water-soluble components after the coagulation of caseins during cheese and yoghurt manufacturing are discharged as a liquid waste stream and is generally termed as "whey". Comprising 80 – 90 % of the total volume of milk, this liquid waste stream contains more than 50% of the milk solids, including valuable WPs and lactose. Due to the high production volumes and the nutritional and functional benefits of whey proteins, the industry is focused on extracting the WPs from the liquid whey streams. World whey output was approximately 240 million metric tons in 2014 and the production rate is expected to increase by 3.5% annually (Lagrange, Whitsett, & Burris, 2015). The latest research shows that WPs

are arguably the most nutritionally valuable proteins available (Sousa, Lira, Rosa, de Oliveira, Oyama, Santos, & Pimentel, 2012). Thus, promoting nutritional markets such as sports, clinical and infant nutrition are driving an unprecedented investment level in dairy production. Whey is proving to be one of the most exciting nutrient sources available today due to the presence of high valued nutritional components, such as high-gelling β-LG, mother's milk equivalent protein α-LA, lactoferrin, and immunoglobulin and as a pre-cursor to the probiotic galactoligosaccharides.

2.3.1 Sweet whey

The whey discharged from the manufacture of hard cheeses, semi-hard cheeses and rennet casein is termed as "sweet whey". Sweet whey is the main stream of whey used so far in manufacturing WP products. The composition of sweet whey is shown in Table 2.2. Compositionally, sweet whey is a balanced whey stream without any extreme salinity or acidity. The total protein content in sweet whey ranges from 0.6-0.8 % and has approximately similar concentrations of β-LG and α-LA to that of milk is observed. Sweet whey contains compositional characteristics close to the physiological characteristics of milk, thus preserving the protein characteristics to a larger extent without much denaturation and aggregation. The pH of sweet whey remains close to the physiological pH of milk, therefore, the effect of acidity and/or alkalinity on protein characteristics is minimum. Thus, the further processing of sweet whey and the successful conversion to shelf stable whey powders is attained.

Sweet whey powder is successfully used as an energy supplement and as a food ingredient due to its excellent functional properties in bakery products, infant formula, confectionaries and desserts.

Table 2.2: Summary of the findings from previous studies on the composition of sweet whey, acid whey and salt whey

Component	Sweet whey	Acid whey	Salty whey	Reference
Total protein (%)	0.4 – 0.6[2] 0.8[3]	0.8[1] 0.6 – 0.9[2]	0.6[3]	[1](Lievore, et al., 2013), [2](Alsaed et al., 2013), [3](Blaschek et al., 2007)
Lactose (%)	2.7 – 4.9	4.2 – 4.9		(Alsaed et al., 2013; Josephson, Rizvi, & Harper, 1975; Lievore et al., 2013)
Titratable acidity (%) (As lactic acid)	< 0.2	> 0.4		(Chegini & Taheri, 2013)
pH	6.0 – 6.5	3.5 – 4.3	5.2 – 5.4	(Alsaed et al., 2013; Kapoor & Metzger, 2004)
Ash (%)	0.6 – 1.9[1]	0.6 ± 1.0[2]	0.4 – 0.6[1]	[1](Alsaed et al., 2013), [2](Lievore et al., 2013)
Salt (%)	0.1 – 0.4		2.6 – 18.9	(Blaschek et al., 2007)
Ca^{2+} (g/L) Cl^- (g/L)	0.5 – 0.7 0.7 – 1.3	0.9		(Jimenez, 2012; Josephson et al., 1975)

2.3.2 Salty whey

Salty whey originates from salted hard cheese manufacturing. In the manufacturing process of salted cheese varieties, an additional salting step is applied after the coagulation of caseins. Therefore, the whey stream discharged after the salting step contains high amounts of sodium salts. The high salinity makes salty whey underutilized due to high processing costs. Compared to sweet whey, salty whey contains approximately 15-20 times more sodium salts (Table 2.2). A comparatively high fat content is in salty whey (Blaschek et al., 2007), mainly

due to the continuous agitation of the cheddar curd post-salting and the increased solubility of fat in a medium with high ionic strength.

Salty whey is rich in lactoferrin and contains comparatively low amounts of β-LG and α-LA as compared to sweet whey (Blaschek et al., 2007). The increased lactoferrin results from the decreased pH during the cheese making process (Dupont et al., 2006). The solubility of β-LG and α-LA is not affected by the high salt concentration of salty whey. However, the solubility of lactoferrin is affected by the increased salt content in salty whey (Blaschek et al., 2007).

Regardless of the distinct composition of salty whey, it is being utilised as an ingredient in different products. For instance, nano-filtration has been used to remove 90% of the salt from salt whey, recovering 80% of the non-salt whey solids which were then incorporated into the sweet whey stream (Gregory, 1987). In another study the permeate was separated and concentrated through nanofiltration and used as a salt addition to cheese varieties (Sanderson, Brady, Whitehead, Oldham, & Brockwell, 1996). Further, salty whey has been used in processed cheese and associated products without any significant impact on quality (Kapoor & Metzger, 2004).

2.3.3 Acid whey

Acid precipitation of casein in manufacturing Cottage cheese, Ricotta cheese and strained yoghurt, primarily Greek yoghurt, yields acid whey as a by-product. Current consumer demand for acid-coagulated products has tripled over the last 5 years and it is predicted that this trend will continue for several years (Elliott, 2013). Therefore, the industry is challenged with an annual production of 1.6 billion liters of acid whey generated as a waste stream.

Compared to sweet whey, acid whey contains similar amounts of protein and lactose, but more salts of calcium and phosphorus (Table 2.2) (Chandrapala et al., 2015). However, acid whey generates through the Greek yoghurt manufacturing process contains comparatively low amount of proteins, due to the protein aggregation caused by the severe heat treatment during pasteurization (Nishanthi, Chandrapala, & Vasiljevic, 2017). Acid whey contains comparatively high concentration of LA, mainly due to fermentation of lactose in milk by lactic acid bacteria. Due to the high solubility of LA, it ends up in acid whey stream during the straining of the casein coagulum. The composition of acid whey varies with the point of origin. For instance, the acid whey derived from soft cheese manufacturing contains comparatively low amount of calcium and phosphates as compared to acid whey obtained from Greek yoghurt. However, lactic acid, the most problematic component in acid whey is present in approximately similar amounts in acid whey obtained from both manufacturing processes.

The protein and non-protein components of acid whey have potential applications in food systems, depending on their purity. For instance, proteins extracted from acid whey can be combined with other milk proteins to use in infant formulas (Rudloff & Lönnerdal, 1992). Lactic acid is a widely used food preservative (Datta, Tsai, Bonsignore, Moon, & Frank, 1995) while isolated lactose can also be used in infant formulas (Chandra, Singh, & Shridhara, 1989). Deacidified and demineralized acid whey are currently used in bakery products (Kosikowski, 1979).

2.4 Manufacturing of whey products

Different types of WP products are available in the market as concentrates, isolates, hydrolysates and demineralized forms of whey protein powders (Figure 2.5). Worldwide production of whey protein products is about 600 000 metric tons per annum (Damodaran, 2008). These products perform different functions in different food systems, which has been identified as the most suitable and efficient way of consuming the WPs. Different types of whey products manufactured from sweet whey are shown in Figure 2.5. Manufacturing of these whey products were predominantly achieved using sweet whey. These whey products are used in the commercial production of beverages including energy drinks, bakery products, confectionaries, desserts and infant formula.

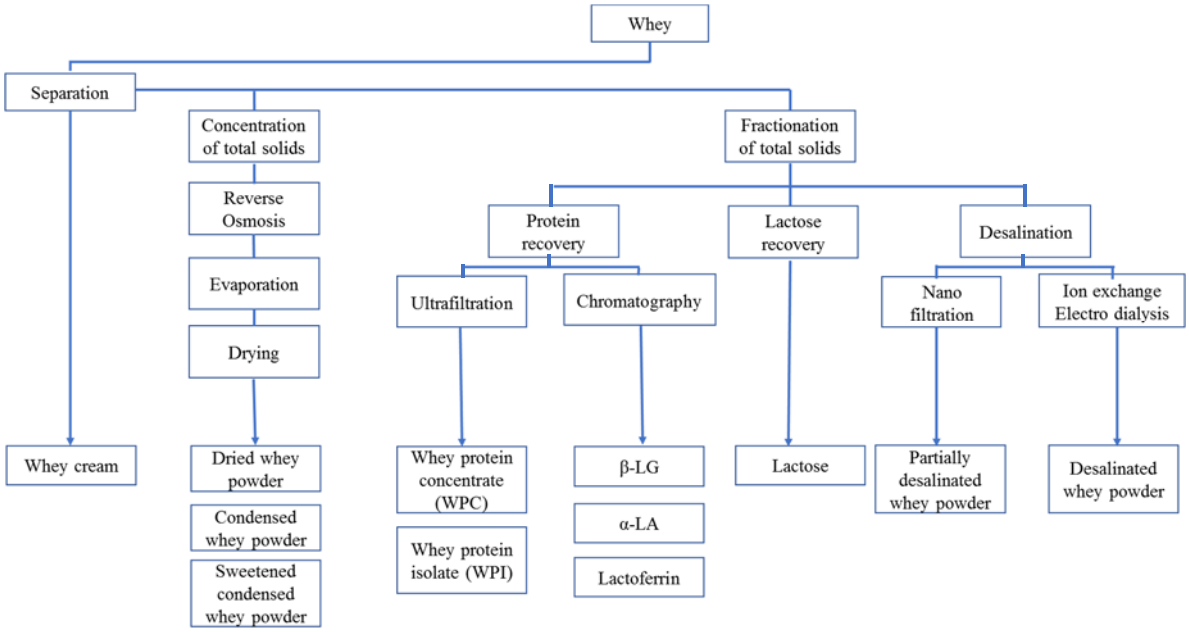

Figure 2.5: Production of different whey products. Sourced from Bylund (2003)

2.4.1 Whey protein concentrate (WPC) and isolate (WPI)

Out of different varieties of whey products, WPC and WPI powders are more popular ingredients used in food manufacturing. The protein content of WPC ranges from 35-80% depending on the extent of protein concentration achieved through membrane processing. Fat and lactose present in WPC cause detrimental effects on some of the functional properties and the overall protein quality. WPI contains at least 90% of proteins on a dry matter basis, thus contains much smaller amounts of non-protein components, mainly 0.2–2.0 % of lactose, 0.2–1.5 % of fat and 0.3–4.5 % of minerals (Morr & Ha, 1993). The main difference between WPC and WPI is the high protein and proportionately low lactose and mineral contents in WPI in comparison to WPC (Table 2.3). Due to the less impact from non-protein constituents, WPI is considered to contain high quality proteins with enhanced functionality.

Table 2.3: Composition difference of WPC and WPI (Abd El-Salam, El-Shibiny, & Salem, 2009; Morr & Ha, 1993) (dry matter basis)

Component	WPC35 (%)	WPC80 (%)	WPI
Moisture	3.0 – 4.5	3.5 – 4.5	4.5
Ash	2.5 – 4.5	3.0 – 4.0	0.3 – 4.5
Protein	34 – 36	80 – 82	90 – 92
Lactose	48 – 52	4.0 – 8.0	0.2 – 2.0
Fat	3.0 – 4.5	4.0 – 8.0	0.2 – 1.5

2.4.2 Manufacture of WPC and WPI

The primary manufacturing steps for WPC and WPI involves separation, pasteurization, concentration and spray drying (Figure 2.6). Producing a good quality WPC and WPI

requires effective separation of cheese fines and the remaining fat from the liquid whey stream with the use of centrifugal clarifiers and separators. Pasteurization is primarily used to reduce or eliminate the microbial load in the whey, that may otherwise harbour dangerous pathogens. The concentration step then concentrates the WPs by removing other non-protein constituents, hence the composition of WPC or WPI differ based on the degree of concentration. Spray drying is the main drying technique applied during the commercial manufacturing of WPC or WPI, with the primary aim being to lower the moisture content of the product to 4% or less.

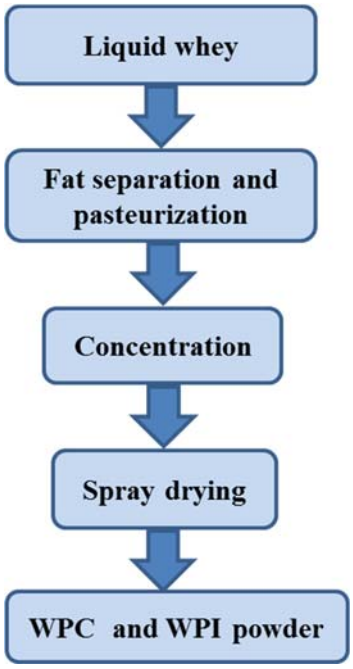

Figure 2.6: Primary steps involved in manufacturing of WPC and WPI

Separation

The separation step is used to extract the remaining cheese fines and remove as much fat as possible from the whey stream (Figure 2.7). Cheese fines are made up of caseins and are removed by centrifugal clarification. Efficient removal of cheese fines is desirable as the

presence of cheese fines can cause negative effects on post-separation, especially clogging the membrane filters, dramatically increasing the cost of production and maintenance.

Liquid whey contains ~0.2-0.4% (w/w) of fat. A cream separator is used to separate the fat from the rest of the whey constituents. Efficient separation is critical to achieve a high efficiency in the following processing steps and to enable customers to meet the end product specifications for their ingredients.

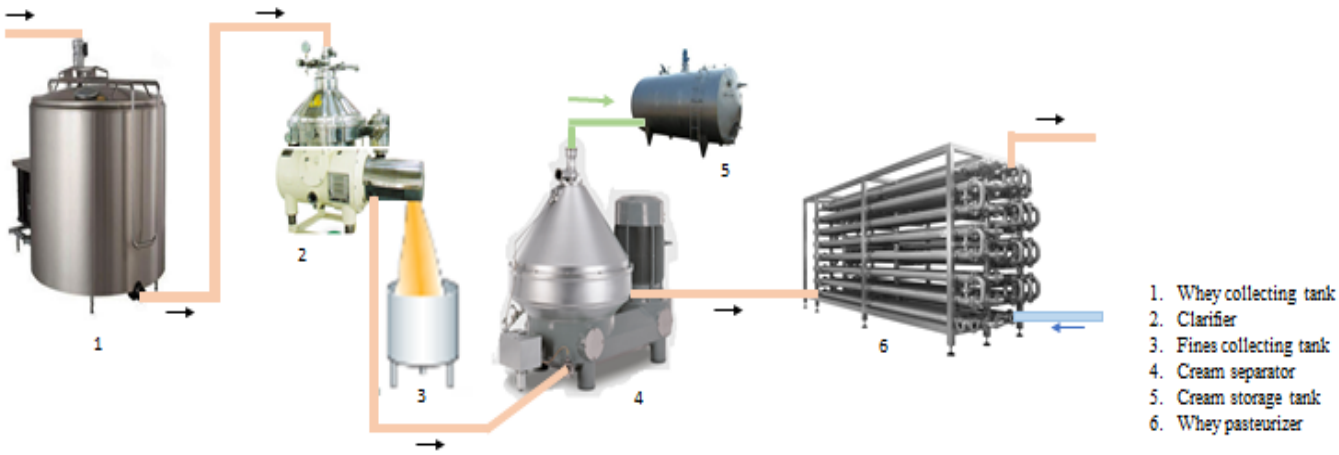

Figure 2.7: Process of whey separation and pasteurization. Sourced from Bylund (2003)

Pasteurization

Whey is pasteurized directly after the removal of cheese fines and the separation of fat due to the necessity of long storage periods and its utilisation in high-quality infant formula and sports nutrition products. High temperature short time pasteurization is applied at most whey processing facilities with a temperature of 72°C for 15 sec. In most whey processing plants, plate heat exchangers are used to pasteurize whey, where counter-current flows of whey and hot water are used (Figure 2.7). Applying this step on liquid whey was found to have no significant impact on the chemical composition, solubility and foaming ability of WPC (Morr, 1987).

Concentration

After separation and pasteurization steps, whey is concentrated primarily to isolate the WPs by removing water and other non-protein constituents. Commercially, the most widely used technique for concentration is membrane filtration, while applying other techniques such as ion exchange chromatography is limited due to high cost associated (Voswinkel & Kulozik, 2011).

Membrane filtration

Semipermeable membranes containing specific pore sizes target the separation of specific whey components into retentate and permeate streams (Figure 2.8). The performance and selectivity of the membrane filtration system was clearly dependent on the processing conditions, such as pH and temperature (Chandrapala, Duke, et al., 2016; Chandrapala et al., 2015), the composition, such as mineral content, and the protein interactions, such as electrostatic and hydrophobic interactions in the feed.

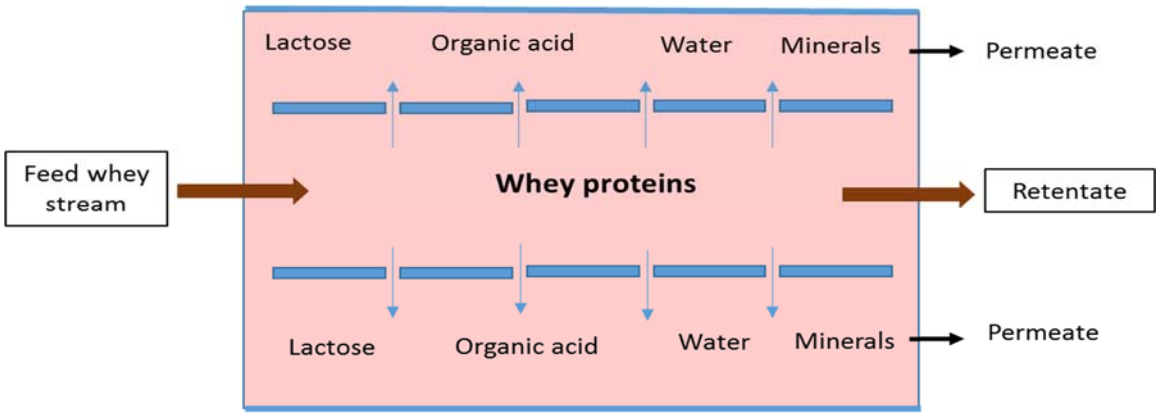

Figure 2.8: Schematic drawing of molecular and flow movement in membrane filtration

There are different types of membrane filtration techniques used by the industry. Microfiltration is a low pressure-driven (100 – 400 kPa) membrane filtration technique,

which uses a membrane with a pore size of 0.1 – 4.0 μm. In the dairy industry, microfiltration is widely used to separate bacteria, remove fat from whey and standardize protein and casein contents.

Ultrafiltration is the main membrane filtration technique applied in the concentration of WPs from liquid whey streams. This is a medium pressure-driven (2-5 bar) membrane filtration technique with a pore size of 0.001–0.1 μm and a molecular size cut off of 1-200 kDa. The typical temperature of ultrafiltration is maintained around 50-55°C (Bansal & Bhandari, 2016). Ultrafiltration combined with diafiltration is also used to remove, mainly water, lactose and minerals, so that selective separation of WPs under mild processing conditions can be acquired. UF of whey results in approximately 24% of total solids, and in combination with diafiltration, the output quality can be increased up to ~38% total solids (Fox & McSweeney, 2003).

Nanofiltration is a medium to high pressure-driven (0.6-4 MPa) membrane filtration process which uses a molecular weight cut-off level in between reverse osmosis and UF. Nanofiltration uses membranes with a pore size ~1 nm. This process allows separation of organics and salts in the molecular weight range ~300–1000 Da (Eriksson, 1988). The separation of minerals by nanofiltration membrane is based on the steric and electrostatic interactions. Divalent ions are rejected by the membrane to a large extent. The mineral content of whey can be reduced by 40–60 % using nanofiltration (Bansal & Bhandari, 2016). Nanofiltration is mainly used for partial demineralization, reduction of the lactose content and the volume of whey during whey processing. For instance, nanofiltration has been used to efficiently separate lactose from sweet whey, which is subsequently crystallized and used as a pharmaceutical ingredient.

Reverse osmosis is another membrane filtration technique driven by high pressure (2-17 bar), which is based on a membrane with a pore size of 0.1 – 2 nm. In principle, only water passes through the membrane layer. Reverse osmosis is normally used during whey processing to reduce the volume of whey.

Membrane filtration of acid whey

Ultrafiltration is successfully utilised in the valorization of sweet whey. However, the application of UF for acid whey is difficult resulting in longer filtration times. The compositional differences of acid whey influence the physico-chemical properties of WPs, lactose and minerals. It was found that the performance and selectivity of membrane systems strongly depend on the pH of the feed (Alkhatim et al., 1998), as pH governs the mineral balance. In acid whey, the presence of LA provides a pH range of 3.5-4.3, where mineral speciation shifts towards the conjugates of Ca (Chandrapala et al., 2015). The insoluble forms of calcium phosphates and lactates deposit on the membrane surface, blocking the pores, leading to membrane fouling. The development of a mineral fouling layer, in turn, attracts the proteins, altering the protein properties, thus forming a dense foul layer, consequently extending the processing time (Figure 2.9). Therefore, it is hypothesised that a pH adjustment is essential in acid whey, prior to membrane filtration. The negative consequence of this type of pH adjustment is in its potential to adversely affect the WP properties, especially their interactional and structural status. However, attempts to manipulate the concentration of minerals and organic acid to improve the separation process of acid whey has been researched recently (Chandrapala et al., 2015). Acid whey contains high amounts of Ca. Calcium interacts with WPs through salt bridging and charge screening, which subsequently leads to intensive protein aggregation. The aggregated proteins have impaired charge distribution,

thus interact electrostatically and hydrophobically with the membranes, limiting further filtration.

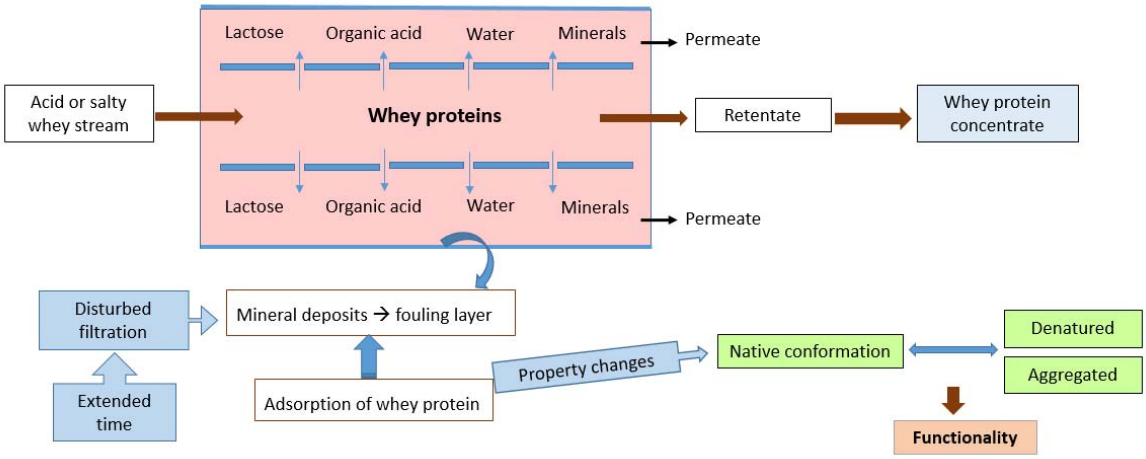

Figure 2.9: Limitations of applying membrane filtration for acid and salty whey liquid streams

Membrane filtration of salty whey

The presence of sodium salts in high concentration in salty whey influences the membrane filtration process, through membrane fouling (Figure 2.9). Most of the membranes contain a negatively charge filtration surface, thus attracting positively charge sodium ions, resulting in the gradual formation of a monolayer of solutes (D'souza & Mawson, 2005). Sodium salts in high concentration denature the proteins through salt bridge formation and charge screening (Zhu & Damodaran, 1994), thus expose previously buried reactive sites such as thiol and hydrophobic sites for polymerization reactions. Denatured proteins contain an unevenly distributed negative charge on the protein surface, thus attract electrostatically with the negatively charged membrane surface. In addition, some of the exposed hydrophobic groups have strong affinities towards the non-polar functional groups of the membrane surface, through hydrophobic interactions (Shi, Tal, Hankins, & Gitis, 2014). Membrane

fouling with denatured WPs cannot be restored without a chemical cleaning process, therefore, delays the filtration process. In addition, pore blockage occurs due to the full or partial closure of membrane pores by the denatured WPs and insoluble salts extending the filtration time. Rapid pore blockage usually happens during the initial stages of filtration (Shi et al., 2014).

Evaporation and lactose crystallization

Liquid WPC achieves a dry matter content of ~ 25-30 % by membrane filtration. Therefore, liquid WPC is further concentrated by evaporation to ~45 – 65 %. At high dry matter content of about 65%, the product can become very viscous, thus no longer flows.

In many processes, evaporation is combined with a lactose crystallization process, where concentrate is spontaneously cooled down to 30 – 40 °C, to initiate the nucleation of lactose crystals. The efficient crystallization of lactose and its removal is essential prior to drying, to avoid stickiness issues during drying. The concentrated liquid WPC is held in specially-designed crystallization tanks for 4 – 8 hours with constant stirring to obtain a uniform distribution of small lactose crystals. Concentrated whey is a supersaturated lactose solution, therefore, under certain temperature and concentration conditions, lactose can crystallize spontaneously, during evaporation.

Unprocessability of acid whey in manufacturing WPC and WPI is mainly governed by the behavior of lactose during the concentration and crystallization steps. The presence of high concentrations of LA negatively affects the lactose crystallization step (Saffari & Langrish, 2014; Shrestha, Adhikari, Howes, & Bhandari, 2006). LA is highly hygroscopic, which in turn induces changes in the structural associations and mobility of adjacent water molecules, preventing the close approach of lactose molecules (Wijayasinghe, Vasiljevic, &

Chandrapala, 2015). Further to that, LA forms hydrated H_3O^+ ions with water via strong H bonds, increasing the energy that is required to remove water from a lactose solution (Wijayasinghe et al., 2015). In addition, the presence of LA has been shown to lower the glass transition temperature and increase the crystallization temperature of lactose (Wijayasinghe et al., 2015) delaying lactose crystallization.

In addition to LA, the presence of high concentrations of calcium and its salts in acid whey influences the crystallization of lactose. Calcium accelerates the growth rate of lactose crystals (Jelen & Coulter, 1973) if the concentration is below 10%. Furthermore, $CaCl_2$ has been found to influence the solubility of lactose, resulting in an increase / decrease of the crystal growth phase of lactose crystallization (Bhargava & Jelen, 1996). The strong electric double layer surrounding the calcium ion interacts with four to six layers of water molecules through dipole-ion interactions, restricting the mobility of water molecules. This restricted mobility of water creates the necessity of high energy requirement for lactose crystallization leading to low crystallization rates (Chandrapala, Wijayasinghe, & Vasiljevic, 2016). In addition, the hydroxyl group of lactose can bind to the hydration layer of calcium, leading to the formation of a calcium-lactose complex. This complexation prevents the orderly arrangement of lactose molecules that is required for crystallization (Chandrapala, Wijayasinghe, et al., 2016).

Spray drying

Concentrated liquid whey, after crystallization and removal of lactose, is spray dried to produce powders (Fox & McSweeney, 2003). Spray drying involves pumping a concentrated liquid feed under pressure through an atomizer. The feed is sprayed into a chamber with hot air to dry rapidly and produce fine powdered particles (Figure 2.10) (Anandharamakrishnan,

Rielly, & Stapley, 2007). The percentage of total solid in the feed, inlet air temperature and outlet air temperature are important variables that needs to be controlled for an efficient spray drying process. The outlet air temperature is the governing factor in regulating the drying rate, which also plays an important role in particle characteristics (Anandharamakrishnan et al., 2007; Anandharamakrishnan, et al., 2008). For instance, Chegini, HamidiSepehr, Dizaji, and Mirnezami (2014) found that by increasing the inlet air temperature by 35°C, the diameter of the particles increased by 15%. Therefore, in commercial spray drying systems, an automatic control is required for faster production, cost minimization, and product quality consistency.

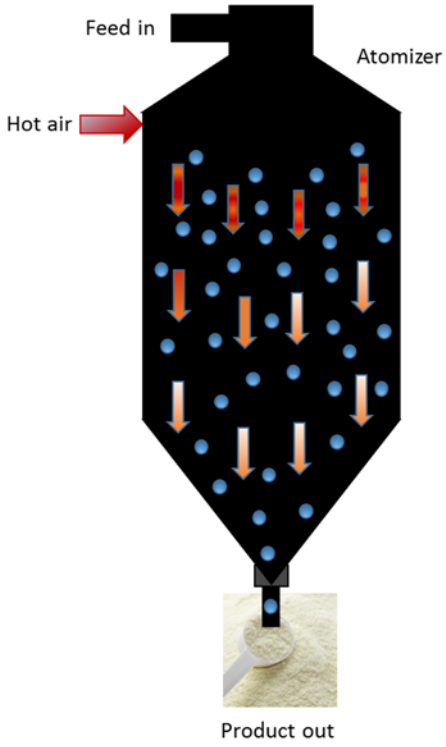

Figure 2.10: Schematic illustration of spray drying process

Spray drying of acid whey

The application of spray drying for the production of acid whey powders is further restricted by the compositional constituents in acid whey. Liquid acid whey is subjected to an uncontrolled thickening during the lactose crystallization step (Mimouni, Bouhallab, Famelart, Naegele, & Schuck, 2007), thus limiting the possibility of drying using a spray dryer. In addition, the highly sticky nature of the resultant acid whey powders, mainly due to presence of lactose and lactic acid, sticks to the dryer and cyclone walls, limiting the drying efficiency (Figure 2.11). Moreover, lumping and caking of the acid whey powder particles during storage due to the hygroscopic nature of LA deteriorate the quality of the powders (Dec & Chojnowski, 2006).

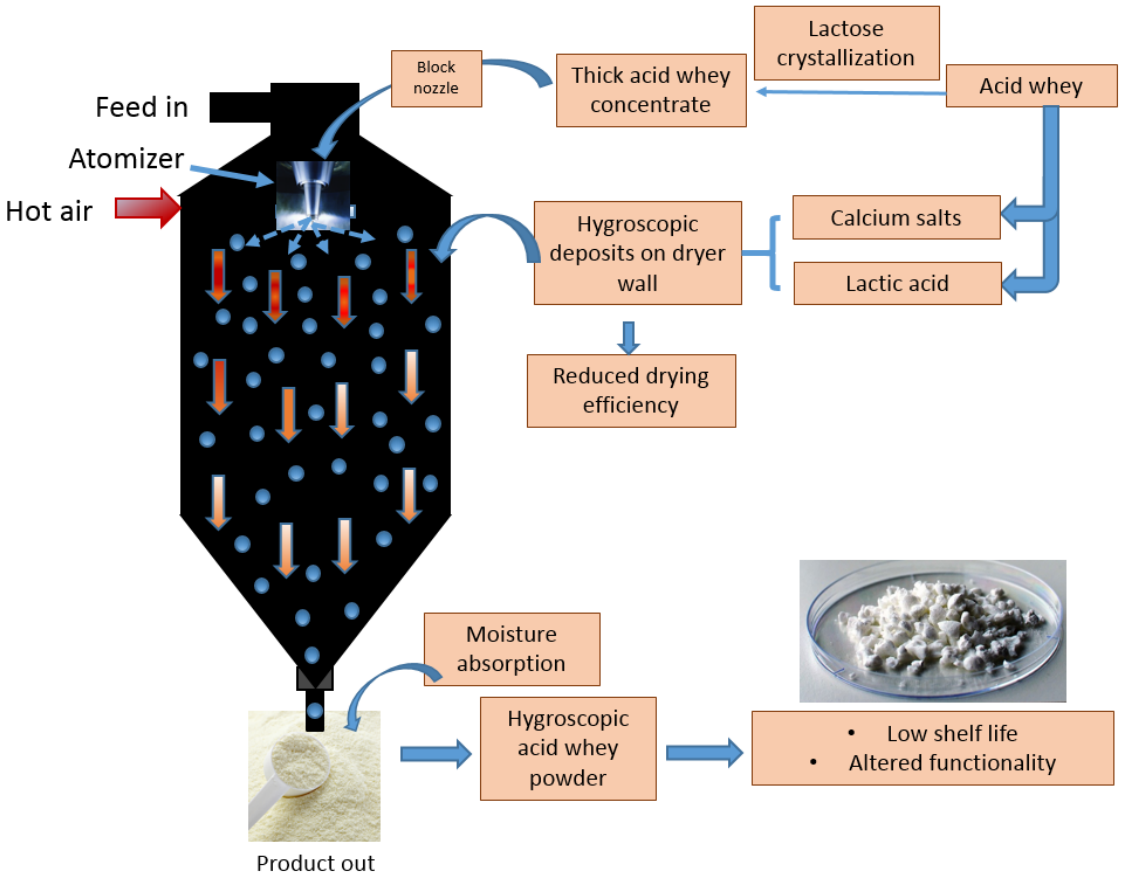

Figure 2.11: Limitations of spray drying acid whey liquid stream

2.5 Denaturation of WPs

The native conformation of a protein contains a net product of various intra- and inter-molecular attractive and repulsive forces, thus is known as the thermodynamically most stable conformation (Damodaran, 2008). WPs are globular proteins with well-defined secondary and tertiary structures. The major alterations in the secondary, tertiary and quaternary protein structures, without breaking the backbone peptide bonds, are known as "protein denaturation". Protein denaturation is possible with any factor that cleaves intramolecular interactions such as hydrophobic, hydrogen, van der Waals interactions and disulphide bonds. Protein denaturation can be reversible or irreversible depending on the extent of the denaturation factors such as heat, ionic strength, pressure and pH. Irreversible denaturation of proteins occurs when the unfolded peptide chain is stabilized by intermolecular disulphide, thiol/disulphide interchange, hydrophobic or electrostatic interactions with other peptide chains. The denaturation can be irreversible in a continuous application of the denaturation factor, after leading to subsequent aggregation (Figure 2.12). The aggregation of unfolded protein molecules results in the association of strands and the formation of a network.

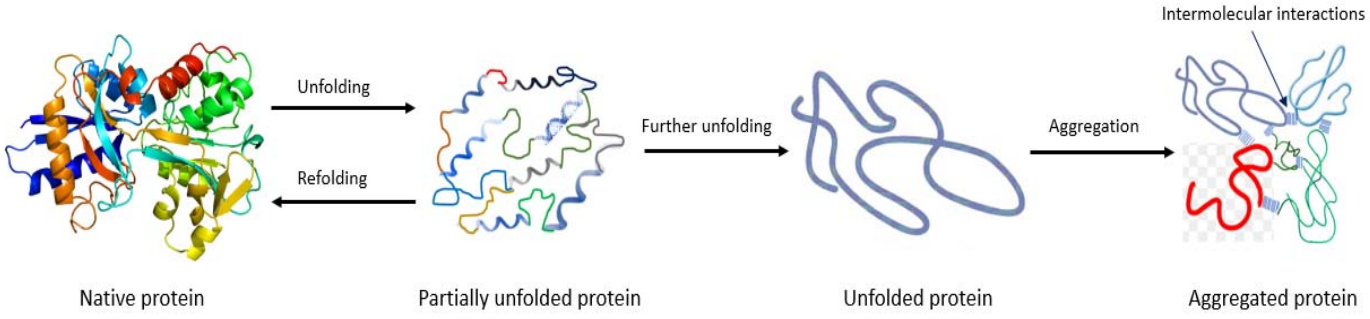

Figure 2.12: Schematic illustration of protein denaturation and aggregation process

2.5.1 Associations of WPs under various commercial conditions

Whey protein-casein associations

Complex and irreversible interactions occur between denatured β-LG and κ-casein in heat treated milk systems. Covalent linking via thiol-disulphide interchange reactions and non-covalent linking via hydrophobic interactions are known to be involved (Havea, Singh, & Creamer, 2001; Jang & Swaisgood, 1990; Oldfield, Taylor, & Singh, 2005). This interaction is affected by the concentration of each protein where, the increased concentration of β-LG resulted an increased ratio of β-LG to κ-casein in the complex formed (Long, Van Winkle, & Gould, 1963). In addition, the temperature of heating was observed to influence the proportion of β-LG involved. For instance, 82% of β-LG interacted with κ-casein when heated at 85°C, while this proportion decreased to 76% when heated at 99°C. Furthermore, the carbohydrate moiety of κ-casein influence the interaction between κ-casein and β-LG. Lesser amounts of β-LG were involved with κ-casein when the sugar content of κ-casein was increased (Doi, Ibuki, & Kanamori, 1981).

The formation of a β-LG trimer at the early stages of forming a complex occurs, followed by the formation of the β-LG/κ-casein tetramer (Haque, Kristjansson, and Kinsella (1987). It was further suggested that hydrophobic interactions govern the initial stages of complexing when heating a 1:1 mixture of β-LG and κ-casein at 70°C under pH 6.8 conditions (Haque & Kinsella, 1988). However, thiol-disulphide interchange interactions become more prominent when heated at temperatures above 80°C, due to the weakening of hydrophobic interactions at high temperatures. At room temperature (25°C), a complex formation between α-LA and κ-casein was observed. However, the complex disappeared with heating to 60-

90°C (Sedmerova, Helesicova, & Sicho, 1972). None of the other WPs were observed to form complexes with κ-casein.

κ-casein is the only casein that was observed to interact covalently with β-LG (Jang & Swaisgood, 1990), although α_{s2}-casein contains a disulfide bond which can participate in interchange reactions. It was suggested that the lack of interaction between β-LG and α_{s2}-casein is due to the interior location of the α_{s2}-casein within the micelle, thus making it difficult for the β-LG to access. In addition, the dimer form of α_{s2}-casein is stable and will not participate in many reactions (Rasmussen, Hojrup, & Petersen, 1992).

Whey protein/whey protein interactions

β-LG forms interactions with other WPs in addition to self-aggregation. α-LA does not readily aggregate or undergo thiol/disulphide interchange reactions, when heated alone, even to 80°C at pH 6.7, predominantly due to the lack of a free thiol in its structure (McSweeney, 2013). However, with the presence of β-LG, the complexation of α-LA occurs rapidly, forming non-native monomers, dimers, trimers or even large aggregates. A non-native β-LG monomer is formed during the initial step, which then reacts with the surface located disulphide bonds of α-LA (McSweeney, 2013; Oldfield, Singh, Taylor, & Pearce, 2000). In addition to the role of disulphide/thiol exchange, hydrophobic interactions also play a significant role during the complexation between β-LG and α-LA (Havea, Singh, Creamer, & Campanella, 1998; Oldfield, Singh, Taylor, & Pearce, 1998; Oldfield et al., 2000). Disulphide-linked β-LG dimers, α-LA:β-LG 1:1 dimers and non-native dimers of both β-LG and α-LA are formed (Havea et al., 2001; Oldfield, 1996). In this reaction, the loss of α-LA occurs rapidly compared to β-LG (Oldfield, 1996). The gels made by a mixture of β-LG and

α-LA are firmer and more consistent as compared to gels made out of β-LG alone. This effect is predominantly due to the increased number of disulphide bonds formed between β-LG and α-LA (Hines & Foegeding, 1993).

Further, the heat-induced interaction of β-LG with BSA exists (Matsudomi, Oshita, & Kobayashi, 1994). The denaturation temperature of BSA is lower than that of β-LG (Patel, Singh, Anema, & Creamer, 2006). BSA and β-LG contains free thiol groups, which make both of these WPs reactive. It has been reported that the response of BSA to heat is almost similar to that of β-LG, although the heat-induced polymerization of BSA starts at a lower temperature (~68 ºC), forming a high number of reactive molecules rapidly. The reaction kinetics between β-LG and BSA follows the second order rate constant (Hines & Foegeding, 1993). The reaction rate and kinetics between β-LG and BSA are predominated by the changes of BSA. This is in contrast to the reaction between β-LG and α-LA, where the reaction rates and kinetics were mostly depended on the changes of β-LG (Matsudomi et al., 1994). Although the aggregation reaction between BSA and β-LG is not affected by the presence of β-LG, the aggregation rate of β-LG is enhanced seven times by the presence of BSA than β-LG alone (Hines and Foegeding (1993). In contrast, the reaction kinetics of BSA was unaffected by the presence of β-LG. However, the formation of soluble aggregates at 75°C through disulphide crosslinks are governed by the reactivity of both β-LG and BSA (Gezimati, Singh, & Creamer, 1996).

BSA also exhibits heat-induced interactions with α-LA. Thiol/disulphide interchange reactions are involved during the aggregation between BSA and α-LA. Matsudomi, Oshita, Kobayashi, and Kinsella (1993) observed a significant increase in gel hardness by the addition of 3% α-LA (w/w) to a 6% BSA solution, which was attributed to the formation of

a more uniform and finer gel matrix due to the participation of thiol/disulphide interchange reactions and hydrophobic interactions. The soluble aggregates formed between BSA and α-LA through thiol/disulphide interchange reactions have lower molecular weights than those formed only with BSA (Matsudomi et al., 1993). Large disulphide bonded aggregates were predominant in this heated mixture of 1:1 BSA and α-LA, along with differently structured monomeric and dimeric α-LA (Havea et al., 2001).

Immunoglobulins (Igs) also exhibits heat-induced interactions with other WPs. Heat treatment similar to high-temperature short-time (72 °C/15s) did not lead Igs to form interactions with other WPs (Bogahawaththa, Chandrapala, & Vasiljevic, 2017). However, heat treatment at 100 °C for 30s showed thiol-disulphide reactions in a mixture of Igs, BSA, α-LA and β-LG, while in a binary mixture with BSA, Igs did not form any covalent interactions (Bogahawaththa, Chandrapala, & Vasiljevic, 2017).

2.5.2 Factors influencing WP denaturation

The globular structures of the WPs are stabilized with various inter- and intra- molecular forces. The stability of the native state is marginal as the net free energy that stabilizes the native conformation against transition to other forms is small, and can be fulfilled by even minor changes in the surrounding medium (Patel, 2007). Thus, WP denaturation and subsequent aggregation is influenced by the environmental factors such as temperature, pH, organic acid, divalent ions, monovalent ions and protein concentration.

Effect of temperature

Heat or thermal energy is one of the major factors that can destabilize the molecular forces, inducing denaturation or unfolding and subsequent conversion to a less ordered conformation. The WP structure undergoes temperature-dependent changes over a wide

temperature range. Heat-dependent unfolding of WPs is characterized by an endothermic heat effect (Singh & Havea, 2003). Heating WPs in a solution or in humid conditions, improves the thermal energy transitions, and thereby increases the molecular mobility, affecting the different elemental components in the polypeptide chain. Subsequent rupturing of inter- and intra-molecular interactions such as, van der Waals, electrostatic, hydrogen, hydrophobic and disulphide results in unordered polypeptide chains. Temperatures up to 60°C cause only minor changes to the hydrophobic interactions and most of these changes are co-operative and reversible. At temperatures above 70°C, hydrogen and hydrophobic bonds rupture, resulting in secondary and tertiary conformational changes. The increase in thermal energy easily exceeds the net free energy of a native molecule, increasing the molecular mobility and the frequency of collision. Collisions occur in the required directions to form permanent thiol-disulphide interactions and disulphide bonds make these changes irreversible. It was found that rupturing of disulphide bonds and the development of free thiol groups begins at 72°C, followed by reaching a maximum at 95°C (Donovan & Mulvihill, 1987).

Different WPs have different responses towards heat. Therefore, the denaturation behavior of different WPs varies. As a result, the thermal transition temperatures of different WPs vary as mentioned in Table 2.4. The onset, peak and end set temperatures of denaturation vary depending on the type of WP, therefore, reflecting the different amounts of energy required for denaturation.

Table 2.4: Thermal denaturation temperatures and enthalpies of WPs. Sourced from Patel (2007)

Whey protein	Onset temperature of denaturation (°C)	Peak temperature of denaturation (°C)	Enthalpy (kJ/mol)
β-LG	78	83	311
α-LA	62	68	253
BSA	64	70	803

Many studies showed that denaturation of β-LG begins at or above 78°C depending on other factors such as protein concentration, pH and ionic strength. Native monomers are converted to non-native monomers during the denaturation of β-LG (Croguennec, 2004). These non-native monomers are reactive which subsequently polymerized via intermolecular thiol-disulphide interchange reactions, into dimers, trimers and tetramers (Havea et al., 2001; Havea et al., 1998). Further, some of the non-native monomers form hydrophobic interactions with the covalently polymerized products (Havea et al., 2001).

The heat-induced polymerization of α-LA has been studied extensively mainly due to its ability to form a molten globule state (Eynard, Iametti, Relkin, & Bonomi, 1992; Havea et al., 2001). Compared to β-LG, α-LA is comparatively heat stable and does not form aggregates permanently via disulphide interchange reactions until it is heated up to 80 °C at pH 6.7 (Matsudomi et al., 1993). High thermal stability of α-LA is gained via the lack of free thiol groups and the existence of Ca bound holo-form (McSweeney, 2013). Due to these two features, there is a high possibility that heat denatured α-LA reconvert to a native protein. However, if α-LA is heated under severe conditions such as 100°C for 30 min, disulphide linked polymers are formed converting α-LA to the molten globule state irreversibly.

BSA is known as the most heat sensitive WP when heated alone at neutral pH. The heat-induced conformational changes of BSA are reversible between 42-50 °C. Further heating up to 60°C, leads the α-helical of BSA to be unfolded irreversibly. Above 60°C, α-helical structures in BSA unfolds completely, exposing the disulphide and thiol groups for aggregation reactions. BSA tends to form a gel if heated to 70 °C while at 80 °C, a self-stable gel is formed (Matsudomi et al., 1993).

Effect of pH

The denaturation and aggregation of WPs shows a strong relationship with the pH of the medium as the kinetics of unfolding and aggregation greatly depend on the electrostatic balance of the proteins. WP surface contains charged groups, in which ionization can vary depending on the pH of the medium. At the physiological pH of milk (pH 6.7), WPs carry an evenly distributed negative charge on the protein surface, thus are stabilized via electrostatic repulsion. Different WPs show different responses to pH as the distribution of polar residues on the polypeptide chains differ (Donovan & Mulvihill, 1987). For instance, at pH 6.7, β-LG is the least stable, while BSA is most stable (Donovan & Mulvihill, 1987). The iso-electric point of β-LG is 5.2, where the polypeptide chain lacks a net charge, hence intra-molecular repulsion is minimum, resulting in a tight protein conformation, which is resistant to unfolding (Donovan & Mulvihill, 1987). At neutral pH, β-LG has a relatively high net charge (McSweeney, 2013), and results in strong intra-molecular repulsions, facilitating unfolding. Exposure of the polypeptide chain at pH above 7.0 increases the tendency for the formation of thiol-disulphide interactions among β-LG molecules. Between pH 7 and 8.5, β-LG undergoes unfolding and refolding series of the protein chain, which is termed as the Tanford

transition (McSweeney, 2013). At extreme alkaline pH, the intramolecular electrostatic repulsion causes swelling and unfolding of the β-LG molecular structure (Damodaran, 2008).

In addition to the electrostatic-steric changes, the availability and reactivity of thiol groups is depended on pH. For instance, the intermolecular disulphide bonds play an important role in WP aggregation at pH 6.4 – 8.0, while the free thiol groups are readily available for reactions at pH 8. Further, at pH 6, non-covalent interactions such as van der Waals, hydrophobic and electrostatic interactions predominate the WP aggregation. At acidic pH, WPs contain an overall positive charge, therefore, thiol-disulphide interchange reactions are unlikely to occur due to the impaired reactivity of thiol groups.

Effect of organic acids

Beside pH, the WP denaturation is also affected by the type of the acidulant present. Common acidulants used in the food industry are lactic acid (LA) and citric acid. LA is a weak acid with a low acid dissociation constant (pK_a). As a result, deprotonation of LA occurs rapidly with the presence of moisture, which in turn decreases the negative charges surrounding the protein molecule. As a result, the electrostatic repulsion between the proteins are disturbed, promoting the attractive forces via van der Waals and hydrophobic interactions (Israelachvili, 2011). A greater tendency to form high molecular weight aggregates through non-covalent interactions when WPs were treated with LA at pH 6 was observed by Dissanayake, Ramchandran, Piyadasa, and Vasiljevic (2013).

Effect of divalent ions

Ca and Mg are the main divalent cations present in whey or milk systems. Ca and Mg ions are considered as chaotropic ions, which destabilize the water structure by solubilizing the non-polar substituents, such as the proteins.

In literature, three mechanisms are proposed in relation to the interaction of divalent ions with WPs. Firstly, divalent ions form ionic-bridges between the negatively charged carboxylic groups of amino acids of adjacent proteins. Secondly, positive charge of the cations shields the charge of the electric double layer around the protein, minimizing the intermolecular electrostatic repulsion and subsequently promotes the attraction forces. Thirdly, the cations, especially Ca, have the ability to directly interact with WPs through their strong binding sites. Previous reports showed that the amount of Ca ions that binds to the β-LG is stoichiometrically proportional to the net charge of the protein (Zittle, DellaMonica, Rudd, & Custer, 1957). Characteristically, α-LA molecule consists of a strong binding site for Ca, where the Ca ion is coordinated by five oxygen atoms of side chain carboxyl groups of Asp_{82}, Asp_{87} and Asp_{88} together with the peptide carbonyl groups of Lys_{79} and Asp_{84}. The binding of salts through strong binding sites, induce local unfolding (McSweeney & Fox, 2013), changing the protein surface properties, which in turn cause aggregation.

The rate and degree of WP conformational changes has been found to be proportional to the concentration and the type of the cations (Zhu & Damodaran, 1994). For instance, the rate and degree of WP aggregation were different with the presence of $CaCl_2$ as compared to $MgCl_2$ at the same concentration (Zhu & Damodaran, 1994). However, the aggregation thermodynamics in response to divalent ions reach an equilibrium after a certain incubation time, achieving a saturated state of aggregation. It appeared that the time taken to reach the

equilibrium-aggregation state is independent of the type of the divalent ion and the concentration up to 0.04 M (Zhu & Damodaran, 1994). WP aggregates that form in response to divalent ions are stable insoluble particles.

In addition, a competition between divalent and monovalent ions to bind to the protein molecule is observed in the presence of a salt mixture. Due to the single positive charge of the monovalent ions such as Na and K, a total replacement of divalent ions attached to WPs does not occur. Only a partial amount of divalent ions replaces with monovalent ions (Jeyarajah & Allen, 1994). Similarly, between two divalent ions, competition exists to bind to the protein molecule. Thus, at high concentrations, Mg has shown the ability to replace all of the bound Ca to β-LG (Jeyarajah & Allen, 1994).

Salt concentration is a main influencing factor for WP aggregation. At low Ca concentrations (0-1.5 mM), the binding affinity of Ca to native β-LG is low (Jeyarajah & Allen, 1994), therefore, exerts a minor influence on the protein conformation. In contrast, at high ionic concentrations, Ca has a significant impact on the WP structure and properties. High Ca concentrations not only cause the immediate onset of aggregation, but also sufficiently interact with the proteins (Ju & Kilara, 1998). Unlike aggregates produced at low Ca concentrations, high Ca presence induces unstable aggregates, which then settle with prolonged incubation (Ju & Kilara, 1998). The ratio of salt to protein is an important key in explaining the above observation. Protein concentration should be sufficiently high to make interactions with Ca during aggregation. Hence, hardly any aggregates observed at a high ratio of salt to protein.

The attachment of Ca and other divalent ions to WP molecules has the capacity to change the surface properties of proteins, including surface hydrophobicity, free thiol content and

electrostatic nature. The charge neutralization effect of Ca alters the native folded state of the protein molecules, exposing the previously buried hydrophobic segments to the surface. Similarly, to the effect of surface hydrophobicity, Ca has the ability to induce conformational distortions in WP molecules that expose the previously buried reactive thiol groups at the sheet-helix interface. Regardless of the native or pre-denatured state of WP, Ca induces local unfolding due to attachment at strong binding sites in WPs, which in turn brings more reactive thiol groups to the protein surface (Jeyarajah & Allen, 1994). The exposed active thiol groups participate in disulphide and thiol-disulphide interchange reactions, leading to a further unfolded state, exposing more free thiol groups. A decrease of reactive thiol content was observed in response to NaCl (Jeyarajah & Allen, 1994) suggesting a formation of an additional disulphide bond instead of exposing the thiol groups.

Effect of monovalent ions

The addition of NaCl to WPCs resulted in the formation of large and dense aggregates, hence decrease the protein solubility (Hussain, Gaiani, Aberkane, & Scher, 2011; Vardhanabhuti, 2008). However, the size and shape alteration was observed in WPs in response to different NaCl concentrations (Hussain, Gaiani, Jeandel, Ghanbaja, & Scher, 2012). WP aggregates acquire thinner and denser particles to more irregular and short (less than 100 nm) particles with low (max. 86 mmol/Kg) to high (517 mmol/Kg) NaCl concentrations. In the absence of NaCl, prevailing negative charge of proteins holds the native conformation through electrostatic repulsion forces, preventing the molecules coming to close vicinity. At low salt concentrations (<60 mmol/Kg), cation concentration is insufficient to screen the negative charges of proteins, therefore, preferential hydration forces drives the hydration of proteins (Hussain et al., 2012). Concentrations below 200 mmol/Kg of NaCl have been shown to form

clumpy aggregates (Hussain et al., 2012) due to the screening of negative electrostatic repulsion and the complete neutralization of negative charges of proteins by Na ions. In concentrations above 200 mmol/Kg of NaCl, dense aggregates were observed (Hussain et al., 2012), that were composed of several aggregated particles. At high NaCl concentrations, spontaneous screening of electrostatic repulsions leads to localized WP aggregations, which collect eventually to form a dense and spherical shaped aggregates, minimizing the surface tension. Compact, spherical aggregates formed at high NaCl concentrations were observed to convert into linear protein molecules making a protein network upon heating (Hussain et al., 2012; Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007).

Structural changes of proteins due to the presence of NaCl alter the surface properties stabilizing the WPs. The surface hydrophobicity of proteins was observed to decrease with the addition of NaCl (Hussain et al., 2012). At high NaCl concentrations, the increased size of protein aggregates limits the surface area, exposing fewer hydrophobic patches. In addition, hydrophobic sites can be buried in the interior of the molecule as a result of the structural modifications occur through ionic-bridging of Na to proteins. Hydrophobic interaction driven aggregates have no or less exposed hydrophobic patches for further bonding.

Effect of protein concentration

Protein concentration itself is not a factor that can induce WP denaturation. However, it facilitates the aggregation step that was initiated by other factors such as heat or ionic strength. Especially at high protein concentrations, heat induced reactions produce multimeric species with high molecular weight aggregates (de la Fuente, Singh, & Hemar, 2002). Therefore, heat-induced structural changes, even at relatively low protein

concentrations, reduce the solubility of WPs regardless of the pH (Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013). It was found that at low protein concentrations, such as 3, 4 or 6% (w/w) and low ionic strength, the protein denaturation step is slower than the aggregation step (Fitzsimons, Mulvihill, & Morris, 2007).

2.6 Kinetics of WP denaturation and aggregation

Reaction kinetics is a mathematical tool that is used to model the WP denaturation. Reaction kinetics for WP systems is based on assessing the amount of native WP concentration, followed by a different chemical or physical reaction resulting in denaturation. According to the different environmental factors discussed in Section 2.5.2 and the type of sample (milk, skim milk, SMUF or buffer) WPs reactivity differs and therefore, results in a wide variation of denaturation kinetics, especially the reaction order and the kinetic constant (Table 2.5). The methods used in calculating the concentration of native protein content includes solubility at pH 4.6, quantitative electrophoresis, DSC and HPLC. A common method is to use the two major WPs, β-LG and α-LA in the estimation of the WP kinetics. Estimation of kinetics is based on several equations to evaluate the reaction rate, order of reaction (n), rate constant (K_n) and activation energy (E_a). The existing literature on WP denaturation kinetic models (Dissanayake, Ramchandran, Donkor, et al., 2013; Oldfield et al., 1998) can be categorized into 1st, 2nd and higher-order models. The majority of these models used WP components either individually or in milk, whey and model systems.

Table 2.5 Summary of kinetic parameters. Sourced from Oldfield (1996)

Whey protein	Medium	Reaction order	Activation energy (kJ mol^{-1})	Analysis method and experimental conditions
β-LG	SMUF, 10% β-LG	2.5	521	DSC (30 to 120°C, heating rate 10°C/min)
β-LG	Phosphate buffer, 0.3% β-LG	1	341	DSC (40 to 170°C, heating rate 20°C/5 min)
β-LG	Distilled water	2	409	DSC (20 to 120°C, heating rate 10°C/min)
α-LA	Skim milk	1	268.6	HPLC (70 – 80°C at pH 6.67 – 6.62)
α-LA	Skim milk	1	164	PAGE, solubility at pH 4.6 (70 – 95°C at pH 6.67)

2.6.1 Kinetics analysis

Fundamentally, the rate equation (1) and the Arrhenius equation (2) are used to calculate the kinetic parameters involved in the disappearance of native WPs (Oldfield et al., 2005). In the literature, several models have been developed using these fundamental equations to assess the denaturation kinetics (Atuonwu, Ray, & Stapley, 2017). Statistical analysis of kinetics data has been conducted using different methods including, linear regression, nonlinear regression and nonlinear regression with adjusted intercept.

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

Where:

C = protein concentration (g kg^{-1})

t = time (days)

k_n = rate constant ((g kg^{-1})$^{(1-n)}$ day^{-1})

n = reaction order

$$k_n = k_0\, e^{\frac{-E_a}{RT}} \qquad (2)$$

Where:

k_0 = pre-exponential term ((g kg^{-1})$^{(1-n)}$ day^{-1})

E_a = activation energy (kJ mol^{-1})

R = universal Gas Constant (8.314 J mol^{-1} K^{-1})

T = temperature (K)

2.7 Physico-chemical changes of WPC powders during storage

Whey protein concentrate (WPC) powders undergo physico-chemical changes during storage. These changes and strongly vary based on the different storage times, temperature and relative humidity (RH) conditions applied. In addition, the composition plays a significant role towards the quality of WPC powders during storage. For instance, WPs, being the major components of WPC powders, are subjected to denaturation followed by aggregation during storage. Further to that, the presence of high concentrations of lactose and proteins can participate in non-enzymatic Maillard reactions. Elevated storage temperature, time and RH conditions accelerate the reaction kinetics of these reactions, thus converting these reactions irreversible. These physico-chemical changes of whey components subsequently affect the physical, structural and thermal characteristics of WPC powders, such as particle size, particle organization, denaturation enthalpy and surface charge.

2.7.1 Protein denaturation and aggregation in WPC powders

WP denaturation and aggregation is a common consequence of the commercial storage of WPC powders, induced by the pH of the product, salt concentration, protein content, temperature, pressure treatments, humidity conditions, processing and storage times. For

certain food systems, such as foams and emulsions, a certain degree of WP denaturation is essential, so that the hydrophobic and hydrophilic segments can align along the interface thermodynamically stabilizing the systems. Food systems require a gel formation such as in desserts, WP denaturation and aggregation is essential, so that a defined network can be formed with a good water holding capacity.

Norwood et al. (2016) found that a proportion of native WPs decreased after 3 months of storage at 40 °C in WPI, with a concomitant increase in the proportion of denatured proteins. These changes were clearly temperature dependent as no changes were observed under low temperatures such as 4 or 20 °C for up to 15 months of storage. At elevated storage temperatures, the flexibility and mobility of WPs increases, resulting in the partial loss of their tertiary structure. Further, storage of WPCs at 60 °C and 22 % RH for one month was found to increase the surface hydrophobicity (Burgain et al., 2016), mainly due to the exposure of previously buried hydrophobic segments to the protein surface after denaturation.

2.7.2 Lactose crystallization

Lactose is a disaccharide consisted of glucose and galactose. Lactose is present in two main forms as amorphous and crystalline. In a WPC powder, both forms of lactose can be present. For instance, in acid-WPC powder, more amorphous lactose is present compared to crystalline lactose. Conversion of lactose from the amorphous to the crystalline state is possible, especially during storage, which leads to negative consequences due to distortion of the particle structures. Crystalline lactose forms a network of capillary interstices in the particle interior, leading to a phase separation between lactose and other components (Kim, Chen, & Pearce, 2009). Crystalline lactose network causes stresses on the oil droplets inside

the powder particles and release free fat onto the particle surface (Kim et al., 2009), subsequently causing particle agglomeration. Storage under elevated temperature and RH conditions promotes lactose crystallization in WPC powders. Crystallization of amorphous lactose in dairy and whey powders is governed by the temperature difference between the storage temperature and glass transition temperature (Morgan, Nouzille, Baechler, Vuataz, & Raemy, 2005). Also, lactose crystallization in dairy systems was found to be influenced by the other components. For instance, Mimouni, Schuck, and Bouhallab (2005) suggested that in the presence of proteins the crystal growth of lactose is reduced. Morgan et al. (2005) further observed a delayed or inhibition in crystallization in WPC even after heating at 60°C under the water activity range from 0.1 to 0.4%. Also, this effect of protein on lactose crystallization was found to depend on the protein concentration, whereas, in the above study, lactose crystallization was delayed in WPC35 while, in WPC60 it was completely ceased. Hydrogen bonding occurred between the polar segments of the proteins and lactose in the dry state (Carpenter & Crowe, 1989), which might cause an interactional competition between protein/lactose and lactose/lactose, thus affect the lactose crystallization. Thomas, Scher, and Desobry (2004) further found that in the presence of β-LG, lactose crystallization is delayed in freeze-dried powders where the protein/lactose ratios were 10/ 90 and 40/60 (w/w). They suggested that the spatial organization of β-LG might be the reason, since β-LG locate on the surface of powder particles and therefore, limit the access of water to amorphous lactose. Further, Haque and Roos (2004) found that lactose crystallization was less delayed in WP powders, compared to caseinate, albumin or gelatin, suggesting that composite dairy systems could still be subjected to lactose crystallization, while pure protein systems cannot. In addition to interactions with protein, Morgan et al. (2005) proposed interactions between

lactose and minerals, which might also impact the crystallization of lactose. Ionic cations and anions can delay the lactose crystallization through altering the structure of water. Presenting as impurities, some ions with low charge density, such as Cl^- lead to perturbation and structure breaking of water molecules while, ions with high charge density such as Ca^{2+}, may lead to reorganization of the water structure in lactose solutions (Bhargava & Jelen, 1996). This type of interactions is possible to occur in mineral rich WPCs, such as acid- and salty-WPCs.

2.7.3 Maillard polymerization

Another type of polymerization reaction that occurs in WPC powders during storage under different storage conditions is lactosylation, or otherwise known as non-enzymatic browning. The loss of nutritional value, formation of flavor compounds, antioxidative and antibacterial compounds, polymerization of proteins and the development of brown color occur. WPs react together with the reducing sugar, lactose, to form polymerized products, which in turn affect the color and flavor of the WPCs. Initiation of the Maillard reaction occurs when lactose reacts with the amino groups of lysine residues, leading to the formation of a Schiff's base. This in turn will undergo Amadori rearrangements to form an Amadori product (lactulosyllysine). During the intermediate stages, Amadori products are broken down to various products such as formic acid, lysyl pyrraline etc. In the final stage, the formation of melanoidins occurs. Dissociation of lysine in the initial stage of the Maillard reaction increases the negative charge of the proteins, thus affecting the electrostatic stability of WPs. Le, Deeth, Bhandari, Alewood, and Holland (2012) reported that six out of twelve lysine residues of α-lactalbumin (α-LA) can be lactosylated during the storage of milk protein concentrates for up to 12 weeks at 40 °C and 84 % RH. It was hypothesized that similar

changes could be expected in WPCs, especially those containing greater lactose contents. These structural modifications and resultant polymers are insoluble aggregates, hence subsequently affect the WP solubility and their other functional properties (Zhou, Liu, & Labuza, 2008).

2.7.4 Fat melting

A considerable amount of fat ends up in the liquid WPC, although fat is removed by centrifugation prior to membrane processing. The residual fat remaining in liquid WPC redistributes within the particle surface and the core during spray drying (Kim, Chen, & Pearce, 2005b). Gaiani, Schuck, Scher, Desobry, and Banon (2007) suggested that during the spray drying, fat is transported towards, and accumulated at the surface. However, other studies shows that, mechanical stress on the particle during spray drying causes fat to migrate towards the surface (Faldt & Bergenstahl, 1995).

The surface fat mainly presents as "free fat" and consisted of high melting triglycerides (Kim et al., 2005b), while a small fraction of surface fat is present as encapsulated fat. The presence of fat on the powder surface renders the surface to be hydrophobic. Further, fat presence on the surface is also readily susceptible to oxidation and the development of rancidity (Hardas, Danviriyakul, Foley, Nawar, & Chinachoti, 2000). Moreover, during storage of dairy powders under elevated temperatures, fat present on the particle surface tends to melt. Gaiani, Scher, et al. (2007) found that the melting point of fat in dried milk was around 25 – 45 °C for low and high-melting lipids. Therefore, at or above the storage temperature of 45°C, large amounts of free fat present on the WP powders melt, thereby altering the particle surface properties. Melted fat subsequently adheres to the surrounding particles, and forms large

agglomerates. Furthermore, the presence of free-fat on the powder surface was found to critically deteriorate the flowability of WPC powders (Kim, Chen, & Pearce, 2005a).

2.7.5 Material transition between powder particle surface and the core

Spray drying leads the components present in liquid WPCs to re-distribute among the particle surface and the core. During the first phase of drying, free water evaporates from the droplet surface. However, at one point, the moisture content becomes inadequate to keep saturated conditions in the surrounding of the droplet, thus the dissolved substances from the saturated solution starts to deposit as a solid layer at the droplet surface. The droplet gradually becomes a particle with a thin solid crust or skin formed at its surface. After this point, the solid crust or skin thickens, restricting further evaporation of water (Birchal, Huang, Mujumdar, & Passos, 2006). The surface composition of a particle is determined largely by the components at the air-water interface during the formation of the droplet during spray drying (Fyfe, 2010). If a component migrates preferentially towards the surface, it greatly influences the surface composition. For instance, both milk fat and proteins accumulate at the air-water interface at the expense of lactose and minerals due to their high surface activity.

Due to the concentration difference of whey components between the crust and the core, a concentration gradient occurs in the particle. During storage, this concentration gradient becomes active and whey components migrate between the surface and the core. It was suggested that solutes with low molecular weights diffuse towards the core faster than the higher molecular weight solutes (Fyfe, 2010). Accordingly, fat and protein tend to remain at the surface, while lactose and minerals travel to the center. Faldt and Bergenstahl (1995) found that storage of lactose rich powder under humid conditions released large amounts of encapsulated fat onto the powder surface, mainly due to the crystallization of lactose. It was

further found that amorphous lactose has the capacity to hold encapsulated fat in its structure, while lactose crystals cannot (Kelly et al., 2015). Therefore, storage conditions that induce the crystallization of lactose, release fat onto the particle surface (Faldt & Bergenstahl, 1995). In addition, these changes affect the microstructure of the particles. For instance, storage of high-lactose/low-protein powder stored under 75% humidity for 4 days, changed the particle organization completely, whereas these changes were absent under low humidity conditions (Faldt & Bergenstahl, 1995).

2.7.6 Changes in particle organization

The aforementioned changes occur in different components of WPC powders ultimately influence the particle organization. Studies that reflect these changes on WPC powders are few, however, this phenomenon has been extensively studied in other dairy powders (Faldt & Bergenstahl, 1995; Faldt, Bergenstahl, & Carlsson, 1993; Kim et al., 2009). The particles of WPC powders are relatively spherical with large 'dents' and have a very smooth surface, reflecting the microstructural characteristics of high protein powders.

Although typical WPC powders have smooth surfaces with pronounced dents, fat extracted surfaces of WPC powders are characterized with very shallow dents (Kim, Chen, & Pearce, 2002). The release of fat to the particle surface occurs during storage, causing particle agglomeration and the formation of a continuous mass (Faldt & Bergenstahl, 1995). Furthermore, many cracks and pores have been observed inside the particles after 6 months storage, due to the migration of encapsulated low-melting triglycerides towards the powder surface (Kim et al., 2009).

As a result of lactose crystallization during storage, crystal-like structures appear on the particle surface, therefore, the globule structure of the particles distorts (Kim et al., 2009). Nijdam and Langrish (2006) suggested that protein accumulation at the particle surface is important to lowers the negative effects of surface lactose. In powders that contain high surface lactose coverage, caking occurs rapidly in humid conditions during the storage above the glass transition temperature.

During storage of WPC powder at high temperatures, moisture is evaporated from the surface, making the surface dry and hard. In addition, the migration of moisture from the core to the particle surface, increases the number of holes appearing on the surface. However, during storage at low temperature and high humidity conditions, the particle surface remains moist and less number of holes form during the storage.

In addition to physical changes in fat, lactose and moisture, protein aggregation occurs during the storage of WPC powders, leading to changes in the particle organization. Particles of WPC powders become larger and acquire irregular shapes if protein aggregation occurs. In the case of extensive aggregation, particle agglomerates form, mainly due to the hydrophobic interactions that forms between particles with aggregated proteins. In the presence of cluster-cluster type aggregation, particles form dense and compact structures similar to cauliflower (Zhang, Arrighi, Campbell, Lonchamp, & Euston, 2016).

2.8 Functional properties of WPC powders

The application of WPC powders as a food ingredient in many food systems is highly desirable due to their ability in acting as gelling, foaming, wetting and emulsifying agents. These functions of WPC powders strongly depend on the physico-chemical properties of WPs, especially their three-dimensional protein structure and related characteristics such as

size, amino acid composition, net charge, hydrophobicity, hydrophilicity and molecular flexibility. As described in Section 2.5.2, these characteristics are governed by the environmental factors such as temperature, pH, ionic strength and protein concentration. Although, functionality of WPCs is governed by the characteristics of β-LG, due to its high proportion, the overall functionality depends on the combined effects of all the other WPs and non-protein components.

2.8.1 Solubility

The solubility of WPC powders is primarily important as it influences the other functional characteristics of WPC such as foaming, gelation and emulsification properties of the food system. WPs are globular in their native form with surfaces covered with hydrophilic residues, thus are highly soluble over a broad range of pH (Zhu & Damodaran, 1994). Water molecules bind to different segments of proteins such as, peptide, amide, hydroxyl and other charged groups (Damodaran, 2008). Thus, solubility primarily depends on the water-binding capacity of the WPs. However, denaturation under varying environmental conditions such as pH (Pelegrine & Gasparetto, 2005), temperature (Dissanayake & Vasiljevic, 2009), ionic strength, protein concentration (Dissanayake, Ramchandran, Donkor, et al., 2013), and other compositional constituents including lactose, fat and organic acids (Dissanayake, Ramchandran, Piyadasa, et al., 2013) can lower the water-binding capacity. Temperature showed a negative correlation with water binding capacity of proteins, as hydrogen bonds are not stable at high temperatures, thus decreasing the solubility (Damodaran, 2008). At pH closer to their iso-electric pH, WPs shows the least solubility due to the lack of electrostatic repulsion (Damodaran, 2008).

The solubility of WPs in a solution primarily depends on the thermodynamic equilibrium between protein-protein and protein-solvent interactions. The physical properties of WPs such as, surface hydrophobicity, hydrophilicity and net charge determine the affinity of proteins to solvent or other protein molecules, thus determines the number of protein-protein or protein-solvent interactions. Therefore, factors that can influence the existence of protein-protein and/or protein-solvent interactions have the ability to determine the WP solubility.

In addition to the environmental factors, the composition of WPCs is also an important parameter that can directly or indirectly affect the functionality of WPCs (Norwood et al., 2017; Tunick et al., 2016). Especially important is the fat in WPC, which tends to concentrate on the particle surface rendering the particle surface hydrophobic, in turn reducing the solubility (Kim et al., 2002). In contrast, WPC powders high in mineral salts can act differently on the solubility of the powders. At low concentrations (0-40 mM), binding of Ca and Mg to WPs through salt bridges and charge screening leads to aggregation, reducing the solubility. In contrast, at high concentrations of salts, additional binding of these cations to protein aggregates leads to re-solubilization (Zhu & Damodaran, 1994). WPC powders rich in lactose and lactic acid are highly soluble due to the hydrophilicity of these two components, which forms strong H-bonds and hydrophilic interactions with water. Protein concentration of WPC powders can differently impact on its solubility, depending on the state of the protein. If proteins are aggregated intensively forming large particles, it affects negatively on the solubility, while WPC powders rich in native proteins showed a high solubility (Dissanayake, Ramchandran, Donkor, et al., 2013).

2.8.2 Heat stability

Poor heat stability of WPC restricts their application in many food systems that require a severe heat treatment. Heat stability is the ability of proteins to withstand thermal processing without detrimental changes such as excessive turbidity, increased viscosity, phase separation, or precipitation or gelation. In WPCs, β-LG and α-LA are the major WPs responsible for the heat instability. Heat stability of WP is improved by extensive WP aggregation, where most of the reactive groups are involved in protein-protein interactions, thus the molecule acquires an inactive state. Therefore, factors that influence the WP aggregation, such as temperature (Dissanayake, Ramchandran, Donkor, et al., 2013), ionic strength, ionic source and the pH of the powder (Dissanayake, Ramchandran, Donkor, et al., 2013) in turn influence the heat stability. For instance, protein aggregation increases with the presence of salt, thereby improving the heat stability. Furthermore, the aggregation of WPs occurs readily in the pH range of 4.8 to 5.3, thereby improving the heat stability. Na and Ca salts, in the presence of LA, deform the WPs present in salty- and acid-WPCs, providing a high heat stability.

2.8.3 Emulsification

WPs are effective surface-active agents because they can lower the interfacial tension between hydrophobic and hydrophilic components in foods. Thus, WPCs are widely used in the formation of oil-in-water and water-in-oil emulsions, due to the stabilizing effect resulting from the formation of a protective barrier around fat droplets, preventing emulsion coalescence. The optimum emulsifying characteristics of WPs depends on the efficiency of diffusion to the newly formed interface, the extent of unfolding and reorienting to lower the interfacial tension and the ability to form a cohesive and viscoelastic film (Monahan,

McClements, & Kinsella, 1993). Emulsifying properties of WPs highly depend on the pH of the solution, as it determines the protein charge, solubility, flexibility and aggregation behavior (Das & Kinsella, 1989). At pH above the isoelectric point (pH>5.2), β-LG has high flexibility, thus provides high stability against flocculation. It was suggested that for WPs stabilized emulsions, conformational flexibility is more important than increased surface hydrophobicity (Shimizu, Kamiya, & Yamauchi, 1981). Heating (Demetriades, Coupland, & McClements, 1997b), ionic strength (Demetriades et al., 1997b), presence of sucrose (Kulmyrzaev, Bryant, & McClements, 2000), sodium ions (Demetriades, Coupland, & McClements, 1997a) or calcium ions (Ye & Singh, 2000) have been reported as factors that influence flocculation, thus the stability of emulsions. WPs have increased negative charge above their iso-electric point, resulting in maximum repulsion, thereby creating a barrier for the droplets to approach each other. This is essential to retard the coalescence and form a stable emulsion. At high temperatures, emulsifying characteristics are impaired through protein aggregation, which in turn lowers the ability to form a stable interface. Furthermore, storage time and conditions influence the emulsification properties of WPC powders. For instance, Hsu and Fennema (1989) found that the storage temperature and humidity should not exceed 20°C and 22%, respectively in order to largely retain the emulsification properties of WPC after 6 months of storage.

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Chapter 3: Properties of whey proteins obtained from different whey streams

Chapter 3 presents a scientific comparison of properties of whey proteins present in different whey streams; sweet, acid and salty whey streams with those of native whey stream that was not subjected to any commercial processing.

The paper entitles “Properties of whey proteins obtained from different whey streams” by Manjula Nishanthi, Todor Vasiljevic and Jayani Chandrapala has been published in the peer-reviewed journal “International Dairy Journal” (2017), 66: 76 – 83. http://dx.doi.org/10.1016/j.idairyj.2016.11.009

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: Manjula Nishanthi Kottahachchi Kankanamge

Signature: [redacted]

Date: 08.09.2017

Paper Title:

Properties of whey proteins obtained from different whey streams

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Manjula Kankanamge	80	Concept development, research question, hypothesis and manuscript preparation
Prof. Todor Vasiljevic	10	Concept development, statistical analysis, artwork preparation and contribute in writing manuscript
Dr. Jayani Chandrapala	10	Concept development, research question, hypothesis, contribute in writing manuscript and submission to journal

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

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Properties of whey proteins obtained from different whey streams

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ABSTRACT

The physio-chemical characteristics of whey proteins (WPs) in sweet, salty, native and acid whey were investigated and compared. WPs from acid whey were characterised by hydrophobically and covalently driven protein aggregation. Covalent aggregation in acid whey consisted of both thiol/disulphide and non-thiol/disulphide mediated reactions. Fourier transform infrared data characterised this protein aggregation as a β-sheet attraction causing subtle changes in the secondary structure. In contrast, WPs in salty whey aggregated via van der Waals, hydrogen, electrostatic interactions and covalent bonds. Both thiol/disulphide and non-thiol/disulphide interactions led to cross-linked β-sheets, disrupting the secondary protein structures. This aggregation exposed hydrophobic segments while oxidising a high number of free thiol groups. The absence of these types of WP aggregation in sweet or native whey highlighted the fact that elevated salt concentration in salty whey or heat treatment applied during production of acid whey are largely responsible for structural differences.

1. Introduction

The residual fraction of milk after coagulation and separation of casein during yoghurt and cheese manufacturing is termed as whey. Whey is generally classified into two groups, “sweet” and “acid”, based on the production process. For instance, acid precipitation of caseins during manufacture of fresh cheeses such as Cottage and Ricotta or Greek style yoghurt yields acid whey, whereas sweet whey originates from the production of rennet type hard cheeses. “Salty whey” is a secondary by product characterised by high salt concentrations applied during the salting step of some cheese varieties such as Cheddar or Colby.

Whey waste streams were initially treated to minimise the environmental impact created by their disposal due to high organic content requiring a high oxygen demand. However, nowadays the primary aim of processing whey waste streams has moved towards concentration and production of whey powders, which are used as food ingredients in many processed foods due to exceptional functional and nutritional properties of whey proteins (WPs). However, it is not expected that all types of whey streams have similar WP properties, as different commercially available whey proteins appear to have different composition and properties (Alsaed et al., 2013; Blaschek, Wendorff, & Rankin, 2007; Dissanayake & Vasiljevic, 2009; Josephson, Rizvi, & Harper, 1975; Wong, LaCroix, & McDonough, 1978) based on the source and processing conditions applied during the upstream processing. Hence, the intrinsic environment created by the compositional constituents such as caseins, minerals and organic acids and processing effects such as heating, acidification or salting are known to alter the surface and structural properties of WPs and thereby affect the final powder characteristics (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Dissanayake, Ramchandran, Piyadasa, & Vasiljevic, 2013; Zhu & Damodaran, 1994).

Whey contains minerals such as calcium (Ca), magnesium (Mg), sodium (Na) and potassium (K) as cations and phosphate, citrate and chloride as anions. Intrinsic ionic environment in liquid whey streams influence the WP properties. Cations interact with WPs by three different mechanisms, i.e., intermolecular salt bridges, electrostatic shielding and direct attachment to binding sites of WPs. These interactions are capable of changing protein conformation, ultimately affecting the functionality of WP powders. As an example, interaction of Ca^{2+} and Mg^{2+} with WPs via formation of salt bridges leads to formation of a viscoelastic film, which may be positioned at a foam interface and provide enhanced foam stability (Zhu & Damodaran, 1994). Also, in the presence of Na^{+} ions, intermolecular repulsion of unfolded proteins is minimised, allowing for greater attraction and creation of a protein network (Hussain, Gaiani, Jeandel, Ghanbaja, & Scher, 2012).

The presence of organic acids such as lactic acid (LA) and citric acid creates an acidic environment in whey streams which may also

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alter the properties of WPs. The presence of LA hinders the hydrophobic interactions of WPs, hence leading to WP aggregation driven by electrostatic, covalent and van der Waal forces (Dissanayake et al., 2013). At or below the isoelectric pH of WP, the number of free thiol groups available for thiol oxidation and disulphide mediated polymerisation of WPs is limited, even after heat treatment. However, above the isoelectric point, irreversible thermal denaturation provides more reactive sites for polymerisation (Monahan, German, & Kinsella, 1995).

Heating is a common processing step used in manufacturing of many dairy products providing for safety and/or modulating protein functional behaviour. Thermal treatments affect the properties of WPs depending on the temperature and exposure time of the treatment. For instance, WPs undergo reversible structural changes mainly due to hydrophobic interactions at mild temperatures (<60 °C), while substantial conformational changes of WPs are observed at or above 80 °C (Chandrapala et al., 2011), due to the participation of reactive thiol groups in thiol–thiol oxidation or thiol–disulphide exchange reactions (Rahaman, Vasiljevic, & Ramchandran, 2015).

Many studies have examined WP properties and functional behaviour using whey protein concentrate and whey protein isolate as the primary sources (Chandrapala et al., 2011; Zhu & Damodaran, 1994), which confounds effects of downstream processing. Thus, assessing properties of WPs in their original aqueous environment would be advantageous in establishing their native characteristics. Availability of information on the conformational and surface properties of WPs derived from different whey waste streams would allow design of suitable methods for mitigating first the problems associated with downstream processing and secondly to propose potential applications for production of WP powders. Although a few studies have focused on analysing the compositional differences between original whey streams (Alsaed et al., 2013; Blaschek et al., 2007; Josephson et al., 1975; Wong et al., 1978), no study has aimed to identify the variations in WP properties among these whey streams. Therefore, the current study aimed to establish the physio-chemical characteristics of WPs present in sweet, acid and salty whey in comparison with those of native whey that has not undergone any commercial processing.

2. Materials and methods

2.1. Materials

Sweet and salty whey streams analysed in the current study were obtained from hard and salted cheese manufacturing plants, respectively, located in the state of Victoria (Australia). Acid whey was obtained from a Greek-style yoghurt manufacturing plant and hence is referred to as “Greek-yoghurt acid whey”. Two batches were obtained on separate occasions, leaving a period of six months in between to account for compositional differences due to seasonal variations of milk. All the chemicals used were of analytical grade and obtained from Sigma–Aldrich Pty. Ltd. (Castle Hill, NSW, Australia) and Bio-Rad Laboratories (Gladesville, NSW, Australia). Ultra-pure water (Milli-Q water, Merck Millipore, Bayswater, Victoria, Australia) was used at all times. Sweet, acid and salty whey samples were first centrifuged at 3500×*g* for 20 min at 20 °C using an Avanti J-26XP centrifuge (Beckman Instrument Australia Pty. Ltd, Gladesville, NSW, Australia) to remove fat and all other coarse particles prior to analysis. As the reference whey stream, which has not been subjected to commercial processing, native whey was derived from raw milk after adjusting pH to 4.6 using 0.1 M HCl to precipitate the caseins and separating the resultant supernatants by centrifugation (Avanti J-26XP centrifuge, Beckman Instrument Australia Pty Ltd.) at 38,400×*g* for 30 min at 20 °C.

2.2. Compositional analysis

Total solid content was determined by oven drying of 1 g of whey at 103 ± 2 °C to a constant weight (AOAC, 2000). Residues obtained by oven drying were combusted in a muffle furnace at 550 °C to determine the ash content (AOAC, 2000). Total solid and ash contents were expressed as a percentage of the original weight of the liquid whey sample.

The Kjeldahl method was used to determine the total nitrogen (TN), non-casein nitrogen (NCN) and non-protein nitrogen (NPN) (AOAC, 2000). A factor of 6.38 was used to convert the protein nitrogen to the protein content of each group of proteins. For the determination of NCN, 50 g of whey was adjusted to pH 4.6 by dropwise addition of 0.1 M HCl. The pH-adjusted mixture was then centrifuged at 5000×*g* for 10 min at 20 °C and the resultant supernatant was analysed. For the determination of NPN, 10 g of whey was mixed with 40 g of 24% (w/w) trichloroacetic acid (TCA) and left for 10 min to ensure full precipitation of proteins. The mixture was then centrifuged at 3000×*g* for 5 min at 20 °C to separate the precipitated proteins and the resultant supernatant was analysed.

Lactose content was determined by a reversed phase-high performance liquid chromatography (RP-HPLC) method (Chandrapala et al., 2015) using methanol in water (85:15) as the mobile phase and a Zorbax (4.6 mm × 25 cm C8) column (Bio-Rad Instruments, Gladesville, NSW, Australia) as the stationary phase. The content of lactic acid (LA) was determined by the HPLC method as described Donkor, Henriksson, Vasiljevic, and Shah (2007) using sulphuric acid (0.01 M) as the mobile phase and an Aminex HPX 87H carbohydrate column (300 × 7.8 mm, Bio-Rad Instruments) as the stationary phase. Mineral composition (Ca, K, Mg and Na) was determined by an inductively coupled plasma (ICP) atomic emission spectrometer (ICP E Multitype, Shimadzu Corporation, Kyoto, Japan) as described by Chandrapala et al. (2015); the wave lengths used for quantification of Ca, K, Mg and Na were 318, 766, 280 and 589 nm, respectively. The colourimetric method described by Chandrapala et al. (2015) was used to determine citrate concentration; absorbance at 428 nm was carried out using a UV spectrophotometer (Biochrom LibraS11, Cambridge, UK), and a standard curve was prepared using tri-sodium citrate with the concentration range 0–1.5 g L^{-1}. Total phosphate and inorganic phosphate contents were determined colourimetrically using a UV spectrophotometer (Biochrom LibraS11) set at a wavelength of 820 nm as reported by Chandrapala et al. (2015); quantification was carried out with a standard curve of potassium dihydrogen phosphate with a concentration range 0–200 mg L^{-1}.

2.3. Protein characterisation

Surface hydrophobicity (S_0) of the protein particles present in whey solutions was measured using a fluorometric assay method (Chandrapala et al., 2011) using 0.008 M ANS (1-anilinonaphthalene-8-sulfonate) as the fluorescence probe. Relative fluorescence intensity (RFI) was measured using a Shimadzu fluorescence spectrophotometer (model-RF5301PC, Shimadzu Corporation) with excitation and emission slits and wave lengths set at 5/5 and 390/470 nm, respectively.

Free and total thiol group content was measured colourimetrically using 2 mM Ellman's reagent [5,5′-dithio-bis (2-nitrobenzoic acid); DTNB] as described by Chandrapala et al. (2011). The absorbance of the yellow coloured compound was measured at 412 nm by a UV spectrophotometer (Biochrom LibraS11) and the net absorbance was calculated by the difference between the

absorbance with DTNB and without DTNB which was then used in calculating the reactive and total thiol concentrations using Eq. (1):

$$Thiol\ concentration\left(\mu mol\ SH\ g^{-1}\right) = (73.53 \times A \times Df)/TS \quad (1)$$

where A is the net absorbance, Df is the dilution factor and TS is the total solids content expressed in mg mL^{-1}.

Surface charge and the particle size distribution of the whey solutions were determined using a Malvern Zetasizer Nano ZSP (Malvern Instruments ltd, Worcestershire, UK) as described by Chandrapala et al. (2015) after appropriate dilutions with skim milk ultrafiltrate (milk permeate). The refractive index and viscosity of milk permeate used in calculations were 1.334 and 0.996 mPa s at 20 °C, respectively (Liyanaarachchi, Ramchandran, & Vasiljevic, 2015).

The Fourier transform infrared (FTIR) spectra of whey were obtained in the range of 4000–600 cm^{-1} using the PerkinElmer Frontier FTIR spectrometer (PerkinElmer, Billerica, Massachusetts 01862, USA) with the combined software of IR Solution (Shimadzu Corporation) Version 1.40. Sample spectra were collected in the absorbance mode after background subtraction. Each spectrum was a result of an average of 16 scans recorded at a resolution of 2 cm^{-1}. The spectra were vector-normalised and smoothened with the aid of the software to recognise the corresponding peaks under the broad amide I region of 1600–1700 cm^{-1} and amide II region of 1500–1600 cm^{-1}.

Native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate (SDS) PAGE (non-reducing and reducing) were performed as reported previously (Patel, Singh, Havea, Considine, & Creamer, 2005). Acrylamide gels (30%) were prepared with or without the addition of 10% SDS for non-reducing SDS or native PAGE, respectively. Samples were mixed with native (Tris base, 0.4% bromophenol blue, glycerol and Milli-Q water) or SDS (Tris base, 0.4% bromophenol blue, glycerol, 10% SDS and Milli-Q water) sample buffers to obtain a final protein concentration of 1 mg mL^{-1}. For reducing SDS-PAGE, 1 mL of sample was mixed with 20 μL of β-mercaptoethanol. Pure β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and casein were used as protein standards for corresponding band identifications. The broad range pre-stained SDS-PAGE standards (Ref. 161-0318, Bio-Rad Laboratories, Hercules, CA, USA) were used to compare the molecular weights. All gels were prepared for imaging by ChemiDoc™ Imaging System (Bio-Rad Laboratories, Gladesville, NSW, Australia) after staining with Coomassie Brilliant Blue and de-staining using a 10% isopropanol and 10% acetic acid solution. The variation of dye absorption by different proteins was considered negligible (Oldfield, Taylor, & Singh, 2005) and the low affinity between Coomassie Brilliant Blue dye and glycomacropeptides (GMPs) (Nakano, Ikawa, & Ozimek, 2007) was assumed to be the same in both non-reduced and reduced samples. For comparison purposes between bands, relative band intensity (RBI) of a particular band was calculated. The RBI was defined as (Eq. (2)):

$$RBI = Band\ intensity/Total\ lane\ intensity \quad (2)$$

2.4. Statistical analysis

The experiments were organised in a randomised blocked design with a type of samples as the main factor and the replications as blocks. Each whey stream was replicated by obtaining two batches, leaving a gap period of 6 months. Each replicate was sub sampled, resulting in at least 4 independent observations ($n \geq 4$) per replicate. The data were analysed using the Statistical Analysis System (SAS Institute Inc., Cary, NC 27513-2414, USA). The ANOVA procedure was used to analyse the significance between four whey streams and means were separated with Tukey's Studentised Range test at a probability level of less than 0.05 ($p < 0.05$).

3. Results and discussion

3.1. Compositional characteristics

Each whey stream has a unique composition (Alsaed et al., 2013; Josephson et al., 1975) that governs its downstream processing and consequently the quality of the end product. Therefore, the chemical differences appear instrumental in governing the process selection and properties of final products. The compositions of the four waste streams are presented in Table 1.

Sweet whey samples contained the highest total protein and WP contents; on the other hand, the protein content was lower in acid whey. Such differences are likely created by manufacturing differences involved in generation of the assessed whey streams. Sweet whey is generated through the production of rennet-type hard cheeses, in which rennet/chymosin is used to initiate coagulation. The chymosin enzyme cleaves the Phe_{105}-Met_{106} bond in κ-casein, yielding *para*-κ-casein and glycomacropeptide (GMP). Most of the GMP would be thus contained in the whey, but the *para*-κ-casein remains attached to the casein micelles and is incorporated into the cheese. The total protein content of the acid whey ranged between 0.23 and 0.25% (w/w), which is low in comparison with the protein content reported by previous study (Chandrapala et al., 2015). While these streams may share a common terminology, it is apparent that, even within a general classification, major compositional differences may exist.

The acid whey obtained for the present study originated from a Greek yoghurt production as opposed to the one from the soft-cheese manufacturing assessed in a previous study (Chandrapala et al., 2015). During Greek yoghurt production, milk is fortified with milk solids and subjected to a severe heat treatment, usually at 90 °C for 5 min (Bong & Moraru, 2014), prior to inoculation with the starter culture, while soft cheese manufacture incorporates a warming step at a very mild temperature, usually at 30 °C. Consequently, WPs denature above 80 °C exposing active thiol groups (Chandrapala, Augustin, McKinnon, & Udabage, 2010) and engaging in thiol–disulphide exchange reactions with caseins, especially κ-casein and α_{S2}-casein (Vasbinder & de Kruif, 2003). Aggregated WPs are subsequently incorporated into the casein coagulum, which depletes the WP content in the acid whey stream.

In addition to protein content, mineral composition also varied substantially among these four whey streams (Table 1). As expected, salty whey contained significantly ($p < 0.05$) higher concentration of sodium (Na) compared with sweet, acid and native whey streams. Such a high content is due to addition of sodium chloride (NaCl) during the salting step of salted cheese varieties (Grummer, Bobowski, Karalus, Vickers, & Schoenfuss, 2013; Lu & McMahon, 2015). NaCl increases a concentration gradient and creates an osmotic condition outside the casein coagulum, facilitating expulsion of whey, process termed as synaeresis. During synaeresis, NaCl deposited on the cheese coagulum is dissolved in whey, increasing the concentration of Na in the waste stream. Concentration of other minerals was slightly elevated in salty whey in comparison with sweet whey (Table 1), although the concentration of Ca and phosphate in acid (0.13% and 0.18%, w/w, respectively) and native whey (both ~0.12%, w/w) was far greater, being almost twice that in sweet or salty whey. Acidification of milk during manufacture of Greek yoghurt via metabolic activity of yoghurt culture or production of native WPs by acid addition lowers pH to isoelectric point (pH ~4.6) of the caseins, which is

Table 1
Compositional analysis of sweet, salty, native and acid whey streams used in this study.[a]

Component	Sweet whey	Salty whey	Native whey	Acid whey
Total solids	6.24 ± 0.6	21.90 ± 1.5	6.95 ± 0.08	5.55 ± 0.4
Ash	0.65 ± 0.04	14.8 ± 0.2	0.77 ± 0.02	0.73 ± 0.02
Total protein	1.04 ± 0.1	0.81 ± 0.04	0.86 ± 0.06	0.24 ± 0.01
Non protein nitrogen	0.01 ± 0.002	0.02 ± 0.003	0.01 ± 0.005	0.01 ± 0.003
Non casein protein	0.98 ± 0.1	0.69 ± 0.03	0.77 ± 0.01	0.20 ± 0.04
Lactose	3.06 ± 0.03	2.82 ± 0.02	3.06 ± 0.04	3.16 ± 0.05
pH	5.38 ± 0.04	5.31 ± 0.005	4.61 ± 0.005	4.52 ± 0.06
LA	0.25 ± 0.03	0.28 ± 0.03	0.20 ± 0.01	0.55 ± 0.01
Ca	0.059 ± 0.0003	0.072 ± 0.0007	0.115 ± 0.01	0.132 ± 0.006
K	0.12 ± 0.004	0.13 ± 0.001	0.046 ± 0.005	0.133 ± 0.007
Mg	0.017 ± 0.001	0.016 ± 0.0001	0.015 ± 0.001	0.017 ± 0.001
Na	0.31 ± 0.004	5.5 ± 0.02	0.014 ± 0.0001	0.021 ± 0.001
Citrate	0.064 ± 0.003	0.072 ± 0.008	0.062 ± 0.006	0.071 ± 0.005
Inorganic phosphate	0.057 ± 0.01	0.1 ± 0.0	0.12 ± 0.0	0.1 ± 0.01
Total phosphate	0.068 ± 0.02	0.088 ± 0.02	0.118 ± 0.01	0.183 ± 0.01

[a] Values are means ± standard deviation ($n \geq 4$); all values except for pH are percentages. Acid whey used in the study was "Greek-yoghurt acid whey".

necessary for coagulation. On the other hand, the calcium phosphate equilibria between the colloidal and aqueous phases of milk is disturbed at low pH, whereby decreasing pH results in re-equilibration of HPO_4^{2-} and PO_4^{3-} in the aqueous phase, leading to dissolution of colloidal calcium phosphate to restore the balance and thus creating high amounts of Ca and phosphates in the whey stream. In contrast, the absence of the fermentation step and subsequent LA production in hard cheese manufacture allows the milk base to maintain the original pH without a significant reduction and thereby minimal dissolution of colloidal calcium phosphate.

3.2. Characteristics of proteins in whey streams

WPs extracted by membrane filtration of sweet whey have widely become a functionally viable ingredient in gelling, emulsifying and foaming (de Faria, Minim, & Minim, 2013). However, the extraction of WPs from acid and salty whey still remains problematic due to fouling of filtration membranes. Varying solubilities of minerals and along with protein–protein interactions result in changes of particle size in the feed streams which in turn causes membrane fouling (Koh et al., 2014). These differences are demonstrated in the present study as a higher average particle size notable for acid (263.8 nm) and salty (213.6 nm) whey in comparison with sweet (178.5 nm) or native (190.3 nm) whey. The ionically active environment in salty whey reduces intramolecular electrostatic repulsion, promoting protein unfolding while reducing intermolecular electrostatic repulsion and allowing WPs to spatially arrange more closely, which in turn promotes intermolecular hydrophobic, van der Waals and thiol interactions that lead to protein aggregation (Jeyarajah & Allen, 1994). The heat treatment applied during the production of Greek yoghurt is another factor contributing to the presence of protein aggregates in acid whey. Above 65 °C, the free sulphydryl group of β-LG can initialise sulphydryl-disulphide interchange reactions and, thereby, enhance protein–protein aggregation (Rahaman et al., 2015). Today, an adequate solution is needed to overcome the problems associated with membrane filtration of acid and salty whey streams. Changing processing conditions during membrane filtration may minimise fouling and enhance the flux; however, these may also impact on the properties of extracted proteins and, in turn, affect their physical functionality.

Physical functionality of WPs is largely governed by their surface properties, primarily based on the presence and availability of hydrophobic segments, free thiol groups and related surface charges. Even subtle changes may result in various interactions on the surface and possibly lead to protein unfolding and subsequent protein aggregation. In this context, three major types of interactions, namely, hydrophobic, disulphide/thiol and electrostatic, are of importance to evaluate to find appropriate membranes for further processing of acid and salty whey streams.

Analysing surface hydrophobicity (S_0) is considered a vital step in predicting protein functionality. Changes in S_0 are mainly influenced by conformational rearrangements in the secondary structure of proteins via induced changes imposed by the environment during processing (Laiho, Ercili-Cura, Forssell, Myllärinen, & Partanen, 2015). As indicated in Table 2, S_0 values of proteins in acid whey were significantly ($p < 0.05$) lower in comparison with those from sweet, salty or native whey stream. The heat treatment applied during Greek yoghurt manufacture triggers conformational re-arrangements due to intermolecular aggregation driven by hydrophobic interactions, hindering hydrophobic amino acid residues within the aggregate structures (Monahan et al., 1995; Rahaman et al., 2015). Furthermore, the presence of comparatively higher amounts of LA (0.55%, w/w) in acid whey as compared with sweet, salty and native whey (Table 1) hinders the hydrophobic interactions suggesting that LA-induced protein aggregation is driven mainly by electrostatic, covalent and van der Waals forces (Dissanayake et al., 2013), thereby acquiring a more stable spherical and dense structure (Xiong, 1992), and limiting the available hydrophobic residues on the protein surface (Rahaman et al., 2015; de la Fuente, Singh, & Hemar, 2002).

In contrast, salty whey had the highest S_0 value of proteins among the four whey streams. The presence of large concentrations of NaCl modifies the electric double layer surrounding a protein molecule (Hermansson, 1979), inducing intermolecular unfolding and exposing previously buried hydrophobic patches. Simultaneously, unmasking and enhancement of negative charge on the protein surface occurs due to exposure of previously buried amino acid residues with negatively charged side chains such as Asp_{129}, Asp_{130}, Glu_{131}, Glu_{134} and Asp_{137}. This is evident by the significantly higher negative charge (−6.7 mV) of salty whey as compared with sweet (−2.8 mV), acid (−3.3 mV) or native (−2.9 mV) whey streams. At high Na^+ concentration such as in salty whey, surface charge and charge density may change, but the surface remains negatively charged (Israelachvili, 2011).

Covalent interactions via thiol–disulphide exchange and thiol oxidation reactions play an important role in varying degree of protein aggregation in different whey streams. The capacity of β-LG to initiate a thiol covalent linkage is due to the free thiol group (Cyc_{121}), while the molecule further has two disulphide bonds (Cys_{66}–Cys_{160}) and (Cys_{106}–Cys_{119}) (Sava, Van der Plancken, Claeys,

Table 2
Surface hydrophobicity, particle size, surface charge and thiol contents of the whey streams.[a]

Whey	Surface hydrophobicity	Particle size (nm)	Surface charge (mV)	Free thiol (μmol g^{-1} protein)	Total thiol (μmol g^{-1} protein)
Sweet whey	82.5 ± 20.5	178.5 ± 6.6	−2.8 ± 0.3	35.1 ± 1.2	45.48 ± 1.3
Acid whey	22.2 ± 5.7	263.8 ± 10.4	−3.3 ± 1.7	3.45 ± 0.8	12.95 ± 0.9
Salty whey	96.1 ± 12.5	213.6 ± 62.5	−6.7 ± 1.2	5.12 ± 0.9	20.45 ± 0.8
Native whey	86.4 ± 21.6	190.3 ± 39.3	−2.9 ± 0.4	4.9 ± 0.9	48.8 ± 0.9

[a] Values are means ± standard deviation (n ≥ 4). Acid whey used in the study was "Greek-yoghurt acid whey".

& Hendrickx, 2005) that can contribute to thiol-disulphide-mediated reactions. In contrast, α-LA contains four disulphide bonds and no free thiol groups. As a consequence, denatured α-LA can be incorporated into the aggregate structure only via thiol–disulphide exchange reactions with denatured proteins that contain free sulphydryl groups.

In the present study, the capacity of thiol -disulphide covalent reactions to occur in proteins present in different whey streams was evaluated by quantification of reactive thiol and total thiol content of the WPs (Table 2). WPs present in sweet and native whey contained greater total thiol content as compared WITH those present in acid and salty whey. However, acid and salty whey streams demonstrated a greater probability of thiol–disulphide exchange reactions as shown by lower amounts of reactive thiol groups. The sodium (Na)-rich environment in salty whey neutralises the intramolecular electrostatic repulsions to a certain extent, which in turn facilitates unfolding of proteins, exposing previously buried free thiol groups on the surface (Jeyarajah & Allen, 1994) and enhancing thiol–disulphide exchange reaction by diminishing intermolecular distances. The heat treatment employed during Greek yoghurt manufacturing is mainly responsible for decreased levels of reactive thiol groups in acid whey. However, in both acid and salty whey streams, thiol–thiol oxidation reactions do prevail compared with native whey, highlighting the fact that high salt content in salty whey and harsh heat treatment conditions during acid whey production promote thiol oxidation (Jeyarajah & Allen, 1994; Rahaman et al., 2015).

3.3. Fourier transform infrared analysis of whey samples

Preserving native structures of WPs is important in processing of whey streams. Isolation, concentration and subsequent drying steps during downstream processing can change native WP structure (Koh et al., 2014) and thereby influence specific functionalities. Therefore, it is of importance to maintain the secondary structure of the WPs in liquid whey waste streams to achieve required functional performances. The particle size data (Table 2) indicated the presence of large aggregated particles in particular whey streams, while S_0, thiol content and surface charge confirmed various interactions prevailing in these systems, likely governed by compositional and environmental differences, as well as manufacturing conditions prior to whey separation. Therefore, it is likely that all these differences may have an impact on secondary structure of proteins present in these streams.

Thus, FTIR analysis was performed (Fig. 1) and amide I region (1600–1700 cm^{-1}) directly relating to the C=O stretching vibrations was analysed. Distinguishable peaks were observed at 1637 cm^{-1} and 1654 cm^{-1}, denoting the presence of intramolecular β-sheet and α-helical structures, respectively, for sweet whey (Adochitei & Drochioiu, 2011; Kong & Yu, 2007; Rahaman et al., 2015). Furthermore, small peaks were observed at 1648 cm^{-1} corresponding to the presence of random coils (Kong & Yu, 2007). The peak observed at 1697 cm^{-1} in sweet whey was assigned to anti-parallel β-sheet aggregated strands denoting a possible aggregation of WPs (Qi, Ren, Xiao, & Tomasula, 2015). Compared with sweet whey, salty whey apparently had no peaks attributed to the native secondary structure, implying the loss of native organisation. On the other hand, a prominent peak was observed at 1683 cm^{-1} (intensity of 0.6604) indicating intermolecular β-sheet aggregation. Hence, the loss of major secondary structural segments (β-sheet, α-helix) was concurrent with intermolecular β-sheet driven aggregation (1685 cm^{-1}) and cross linking (1618–1620 cm^{-1}) (Kong & Yu, 2007), which confirmed some of the reasons for the presence of protein aggregates in salty whey.

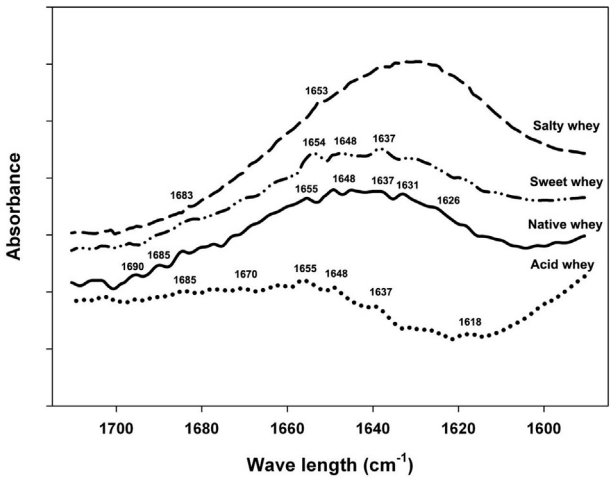

Fig. 1. Original spectra of the amide I region (1600–1700 cm^{-1}) for whey proteins from four whey streams.

The FTIR spectrum of native whey consisted of peaks denoting the presence of β-sheets at 1633 cm^{-1} and 1637 cm^{-1}, random coils at 1648 cm^{-1}, α-helix at 1655 cm^{-1} and β-turns at 1690 cm^{-1}. However, compared with sweet whey, a new, less intense peak was observed at 1626 cm^{-1}, suggesting a partial unfolding of β-LG (Rahaman et al., 2015), likely due to acid-induced structural changes of proteins. In comparison with native whey, FTIR spectrum of acid whey is characterised by less intense peaks at 1648 cm^{-1}, 1655 cm^{-1} and 1670 cm^{-1}, suggesting the presence of random coils, α-helices and β-turns, respectively, to a lesser extent and indirectly indicating the presence of protein aggregates. Similarly, to native whey, acid whey showed a peak at 1685 cm^{-1}, indicating intermolecular β-sheet aggregation, likely induced by low pH. Furthermore, a certain level of protein aggregation was confirmed, with a distinct peak at 1618 cm^{-1}, representing intermolecular cross linking of β-sheets that occurs due to extensive aggregation (Lefèvre & Subirade, 1999). The absence of this peak in native whey demonstrates that additional aggregation in acid whey was a consequence of heat treatment applied during the production of Greek yoghurt.

3.4. Polyacrylamide gel electrophoresis of whey streams

Effects of compositional variations and processing conditions on surface properties of WPs and subsequent aggregation via various

interactions are evident from our results. Therefore, further analysis was conducted to investigate structural and molecular properties of these aggregates and the type and extent of covalent and non-covalent aggregation using native and SDS-PAGE under reducing and non-reducing conditions (Fig. 2). Comparisons between native PAGE and SDS-PAGE under non-reducing conditions show involvement of hydrophobic interactions, while the aggregate bands in reducing SDS-PAGE patterns are entirely due to non-disulphide covalent interactions. Thus, the differences between protein bands under reducing and non-reducing conditions show the disulphide-linked covalent interactions.

Native gel patterns of acid whey [relative band intensity (RBI) = 0.27], when compared with that of native whey (RBI = 0.04), showed a large quantity of high molecular mass aggregates (>250 kDa) that did not enter the gel and remained in the sample well (Fig. 2A). Levels of aggregates of ~250 kDa that remained on top of the resolving gel (Patel et al., 2005) were also approximately five times higher in acid whey (RBI-0.45) than in native whey (RBI = 0.09). In parallel, a reduction of 85% of β-LG in acid whey was observed in comparison with native whey, implying that this protein was one of the main constituents in these aggregates. The intensity of the bands corresponding to aggregates with Mw of ~250 kDa was reduced by 71% and 41% under non-reducing conditions in acid and native whey, respectively, as compared with native PAGE. This highlights the fact that more hydrophobically mediated aggregates were present in acid whey, which was consistent with the S_0 data, and indicated significant ($p < 0.05$) electrostatic shielding of whey proteins in acid whey. Hydrophobic segments of WPs must have induced hydrophobic interactions, prompting further folding and aggregation, burying hydrophobic segments into the hydrophobic core, and hence lowering availability of hydrophobic segments on the surface. Reducing SDS-PAGE patterns (Fig. 2C) showed that the band corresponding to 250 kDa was completely absent in both acid and native whey. Concurrently, bands corresponding to 150 and 133 kDa were present in native whey (RBI = 0.09) and acid whey (RBI = 0.27), respectively. Furthermore, β-LG levels increased by 31% and 14% in acid and native whey, respectively, while α-LA levels did not change. This implies that, in acid whey, β-LG forms thiol-disulphide-mediated covalent interactions creating high molecular mass protein aggregates. The prevalence of disulphide linkages in proteins present in acid whey was also evident from the total and free thiol data in the current study (Table 2). However, in both acid and native whey, β-mercaptoethanol treatment was not adequate to fully dissociate the protein aggregates to its monomers, denoting the occurrence of non-thiol/disulphide covalent linkages. In particular, the occurrence of a high concentration of partially reduced aggregates in acid whey as compared with native whey indicates the presence of more non-thiol/disulphide covalent cross links, which coincides with the FTIR spectrum of acid whey.

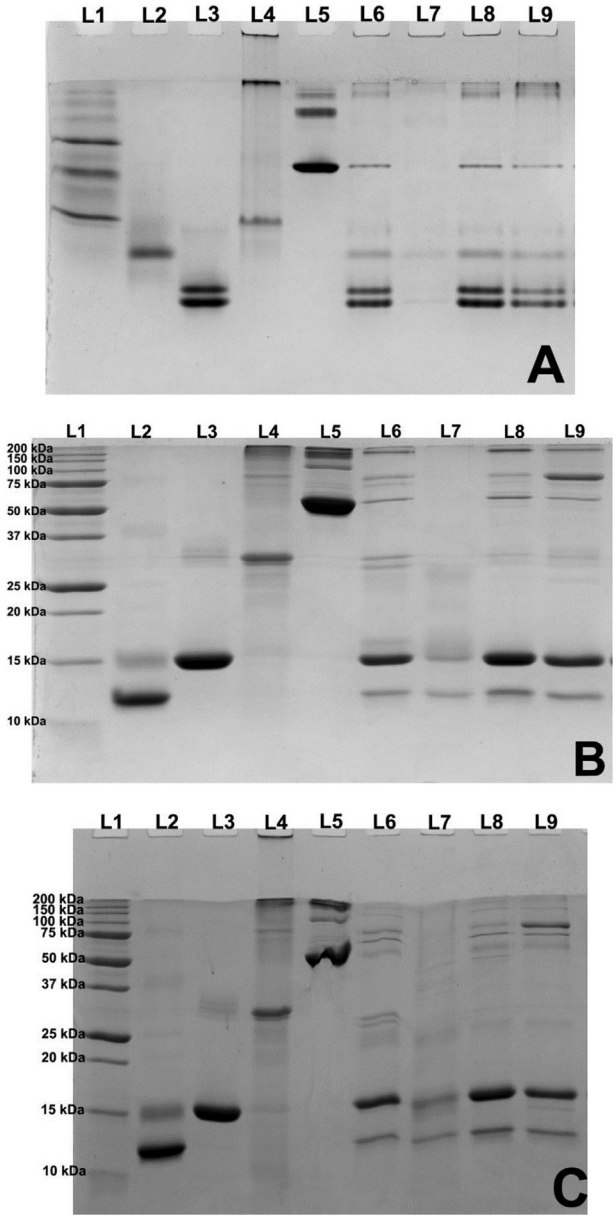

Fig. 2. Native- (A), non-reducing SDS- (B) and reducing SDS- (C) PAGE analysis of whey proteins derived from four whey streams. Lanes are: L1, molecular mass markers; L2, α-lactalbumin standard; L3, β-lactoglobulin standard; L4, casein standards including α_S-, β- and κ-casein; L5, bovine serum albumin standard; L6, native whey; L7, acid whey; L8, sweet whey; L9, salty whey.

Sweet and salty whey consist of similar quantities of protein aggregates of >250 kDa (RBI = 0.04) under native conditions (Fig. 2A). However, this quantity was five times higher in salty whey (RBI = 0.15) than in sweet whey (RBI = 0.03). In parallel, the level of β-LG appeared to be 15% lower in salty whey as compared with sweet whey under the same conditions. Modifications of dielectric protein charge in presence of elevated concentrations of Na^+ may greatly impact on major WPs, leading to unfolding and aggregation as also indicated by larger particle size (Table 2). In non-reducing SDS gels, sweet (RBI = 0.1) and salty whey (0.09) contained protein aggregates corresponding to 250 kDa (Fig. 2B). However, the intensity of this band decreased by 56% under non-reducing conditions as compared with native PAGE, implying that 56% of these aggregates were non-covalent bonded. Non-covalently driven WP aggregation in salty whey must have been predominantly driven by van der Waals attraction. High concentrations of Na^+ modify the negative charge around the WPs, weakening the repulsive forces, and hence strengthening the van der Waals attractive forces driving aggregation. When the surface charge is low (−6.7 mV) and ionic strength is high (Na^+ ~ 1 M), attractive forces prevail over all distances and induce rapid aggregation (Fennema, Damodaran, & Parkin, 2008).

Since WPs in salty whey had the highest S_0 compared with the other three whey streams (Table 2), this may also imply that aggregation was less likely to be governed by hydrophobic interactions. Interestingly, a non-native protein band appeared with of ~65–90 kDa in both sweet (RBI = 0.04) and salty whey (RBI = 0.2) more likely formed via covalent disulphide links. After reducing with β-mercaptoethanol, both in sweet and salty whey,

protein aggregates of ~250 kDa were visible with reduced intensities. Accordingly, RBI of the protein aggregates declined from 0.087 (non-reduced) to 0.056 (reduced) for salty whey confirming that ~36% of protein aggregates in salty whey were cross linked via thiol-disulphide mediated reactions. Concurrently, appearance of bands corresponding to 138 kDa with a RBI of 0.02 was observed in both sweet and salty whey under reduced conditions, indicating the presence of non-thiol/disulphide covalent cross links other than thiol/disulphides bonds. Non-native protein bands appearing in non-reducing gels for sweet and salty whey of ~65–90 kDa remained unchanged even after reduction with β-mercaptoethanol, implying that aggregation was predominantly driven by non-thiol/disulphide mediated cross links. FTIR spectra for sweet and salty whey suggested a possible intermolecular β-sheet aggregation. The presence of salt in whey might imbalance the dielectric environment of proteins, leading to protein unfolding and subsequent exposure of segments for covalent links.

According to the protein characteristics obtained by FTIR and PAGE analysis, it appears that covalent and hydrophobic interactions have driven the WP aggregation in acid whey, while van der Waals, hydrogen and electrostatically driven WP aggregation predominated in salty whey. At lower pH values, negative ion density is in proximity to Arg_{148} on dimer-interface of β-LG, while at high pH the ion density shows a scattered distribution on the α helix and around $Arg_{148,}$ due to the neutralisation by the negatively charged amino acid residues such as Asp_{129}, Asp_{130}, Glu_{131}, Glu_{134} and Asp_{137}. However, due to high ionic strength as in salty whey, the ion density is localised around the α-helix and surrounds positively charged Lys_{135}, Lys_{138}, and Lys_{141} residues (Mercadante et al., 2012). In addition, the Glu_{114} residue surrounded by a strong positive ion density projects towards the dimer surface at high pH and high ionic strength. However, the electrostatic potential of the protein indicates an overall negative charge, as evidenced by surface charge data. Thus, this positively charged region associated with the dimer surface and the negative region associated with the main α-helix governs formation of more electrostatically aggregated particles in the salty whey stream as compared with acid whey. Furthermore, the decrease in pH consequently leads to a decrease in surface tension of the proteins, which then again can be attributed to negligible electrostatic repulsions and thereby favour stronger attractive hydrophobic interactions, such as in case of acid whey.

4. Conclusion

Compositional variations and applied processing methods impact the properties of WPs. Lower pH, applied heat treatment or change in ionic strength induce conformational changes in proteins and facilitate aggregate formation. WPs present in acid whey aggregated via hydrophobic interactions and covalent linkages. Both thiol/disulphide mediated reactions and non-thiol/disulphide reactions appeared responsible for aggregate formation via covalent interactions in acid whey. Aggregation of WPs in acid whey was characterised by intermolecular β sheet aggregation and intermolecular cross linking and most importantly had a less influence on the secondary protein structure. In contrast, in salty whey, WPs aggregated via strong covalent bonds and weak van der Waals, hydrogen and electrostatic interactions, exposing the hydrophobic sites to the surface. The covalent aggregates seemed to be created by both thiol/disulphide mediated and non-thiol/disulphide mediates reactions and were characterised by intermolecular β sheet aggregation and loss of other native secondary structure descriptors. Apparently, different additional processing approaches appear to be required to convert salty and acid whey to functionally viable whey streams similar to sweet whey. Demineralisation may be needed for salty whey to remove the salts, while nanofiltration can be incorporated for acid whey to remove LA. These two processing approaches may adjust acid and salty whey streams more compositionally comparable with sweet whey, thus enabling production of whey powders with similar functionalities as sweet whey powder.

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Chapter 4: Compositional and structural properties of whey proteins of sweet, acid and salty whey concentrates and their respective spray dried powders

Chapter 4 presents a study on how compositional variations of native, sweet, acid and salty whey streams influences their surface and structural properties of whey proteins during concentration and spray drying.

The paper entitles “Compositional and structural properties of whey proteins of sweet, acid and salty whey concentrates and their respective spray dried powders” by Manjula Nishanthi, Jayani Chandrapala and Todor Vasiljevic has been published in the peer-reviewed journal “International Dairy Journal” (2017), 74: 49 – 56. http://dx.doi.org/10.1016/j.idairyj.2017.01.002

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: Manjula Nishanthi Kottahachchi Kankanamge

Signature: [redacted] **Date:** 08.09.2017

Paper Title:

Compositional and structural properties of whey proteins of sweet, acid and salty whey concentrates and their respective spray dried powders

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Manjula Kankanamge	80	Concept development, research question, hypothesis and manuscript preparation
Dr. Jayani Chandrapala	10	Concept development, research question, hypothesis and contribute in writing manuscript
Prof. Todor Vasiljevic	10	Concept development, statistical analysis, artwork preparation, contribute in writing manuscript and submission to journal

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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Compositional and structural properties of whey proteins of sweet, acid and salty whey concentrates and their respective spray dried powders

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ABSTRACT

The influence of physiochemical characteristics of whey concentrates obtained by ultrafiltration of acid and salty whey streams on the surface composition, particle organisation, secondary structures and protein interactions of the respective spray dried whey powders was investigated. Their properties were compared with those of native and sweet whey. Acid whey concentrate demonstrated characteristically low surface charge, high surface hydrophobicity, high average particle size and high thiol activity compared with sweet and native whey concentrates. Salty whey concentrate was characterised by low surface hydrophobicity, high thiol activity and low average particle size. Surface characterisation of whey powders revealed protein-rich surfaces for all whey powders while those in salty whey were highly hydrophobic. Protein characteristics of native and sweet whey powders largely followed those of concentrates. In contrast, protein characteristics of the acid and salty whey powders largely changed from those of the concentrates.

1. Introduction

Manufacturing processes employed in the production of certain types of yoghurt, caseinates and many cheese varieties separate liquid whey as a waste stream from a bulk of the total solids. This waste stream is rich in whey proteins (WPs) (20% of total milk proteins), lactose and minerals, while the composition largely varies depending on the type of whey usually classified as sweet, acid and salty (Blaschek, Wendorff, & Rankin, 2007). Whey is rich in carbonic compounds; therefore its disposal is difficult due to potential environmental issues. Hence, whey utilisation becomes a plausible solution as it reduces the environmental impact while adding a value to a resulting product. Valorisation methods applied to liquid whey streams involve WP concentration step through membrane processing mainly ultrafiltration (UF) prior to drying.

Currently, sweet whey is subjected to UF yielding a whey concentrate, which is either used as a food ingredient itself or subjected to spray drying to produce a whey powder with an extended shelf life. Exploring acid whey as a possible source of WPs started recently, but it advances at a slow pace. Characteristically high acidity and mineral content in acid whey cause fouling of the UF membranes (Chandrapala et al., 2016), which subsequently extends the duration of the UF process. In addition, the presence of highly hygroscopic lactic acid (LA) renders spray drying difficult by promoting formation of powder agglomerates and sticky deposits in the dryer. At present, only limited knowledge (Liao & Mangino, 1987) is available on the physiochemical properties of WPs in acid whey concentrates (AWC) and their respective spray dried powders. Further, no study has been conducted on the physicochemical properties of WPs present in salty whey concentrates and their respective powders due to rapid membrane fouling. Therefore, clear scientific evidence that would facilitate development of effective utilisation methods for acid or salty whey is absent.

The current study investigated how the origin of WPs present in different whey streams (native, sweet, acid and salty) influences their surface and structural properties during concentration and consequently sprays drying. Fundamental knowledge gained in this study would facilitate greater re-utilisation of acid and salty whey streams, which in turn may minimise the waste disposal.

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2. Materials and methods

2.1. Materials

Acid whey was provided by a Greek yoghurt manufacturing facility (Melbourne, VIC, Australia). Ultrafiltration (UF) of acid whey was carried out using two Koch 10 kDa PES membranes with a 3838 HpHT HFK131 V spacer membrane (5 m^2 surface area each) under a transmembrane pressure of 3 bar while the flow rate of the feed was maintained at 7000 L h^{-1} to achieve a concentration factor of 1.6. To further concentrate proteins, acid whey concentrate (AWC) was additionally subjected to two diafiltration steps under the same processing conditions, adding 100 L of water at each step.

Salty whey was provided by Warrnambool Cheese and Butter Factory (Warrnambool, VIC, Australia). Salty whey was ultrafiltered achieving a concentration factor of 1.2 using the same method mentioned above for acid whey, without the additional diafiltration step.

Raw milk for the preparation of native whey concentrate (NWC) was provided by Parmalat Australia (Rowville, VIC, Australia). NWC was prepared by a two-step process. In the first step, pasteurised (73 °C, 18 s) and skimmed (GEA Westfalia separator AG D 59302, Oelde, Germany) milk was microfiltered (MF) using three 1.4 μ Isoflux ceramic filters (0.35 m^2 surface area each) (Tami industries, Z.A. Les Laurons, Nyons Cedex, France) under a transmembrane pressure of 0.83 bar to remove milk fat, somatic cells, bacteria and caseins. In the second step, the MF permeate was ultrafiltered using the process applied in the acid whey concentration without diafiltration.

Two batches of whey concentrates were prepared using two batches of each feed obtained on separate occasions with a gap period of six months in between to account for compositional differences due to seasonal variations. Similarly, two batches of sweet whey concentrate (SWC), which were 6 months apart in production dates, were obtained from Warrnambool Cheese and Butter Factory.

All four whey concentrates were spray dried using a GEA Niro Mobile Minor spray drier equipped with a Niro rotary atomiser. Inlet and outlet temperatures were maintained at 210 °C and 80 °C, respectively. Spray dried powders were sealed in stand-up metalised barrier pouches and kept frozen until further analysis.

The chemicals used in the study were of analytical grade and obtained from Sigma–Aldrich Pty. Ltd. (Castle Hill, NSW, Australia) and Bio-rad Laboratories (Gladesville, NSW, Australia). MilliQ water (Milli-Q water, Merck Millipore, Bayswater, VIC, Australia) was used at all times.

2.2. Compositional analysis

Determination of total solids, ash, total and whey proteins, and non-protein nitrogen was conducted according to the methods stated in AOAC (2000). Lactose content was determined by reverse phase high performance liquid chromatography (HPLC; Chandrapala et al., 2015). Content of lactic acid (LA) was determined by HPLC (Donkor, Henriksson, Vasiljevic, & Shah, 2007). Mineral composition (Ca, K, Mg and Na) was determined using an inductively coupled plasma (ICP) atomic emission spectrometer (ICP E Multitype, Shimadzu Corporation, Kyoto, Japan) as described previously (Chandrapala et al., 2015). Total phosphate and inorganic phosphate contents were determined colorimetrically using a UV spectrophotometer (Biochrom LibraS11, Cambridge, UK) set at the wavelength of 820 nm (Chandrapala et al., 2015).

2.3. Protein characterisation

Surface hydrophobicity of the protein in whey concentrates was measured using a flurometric assay method (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011) with 0.008 M ANS (1-anilinonaphthalene-8-sulfonate) solution as the fluorescence probe. Fluorescence Intensity (RFI) was measured using a Shimadzu Fluorescence Spectrophotometer (model-RF5301PC, Shimadzu Corporation) at the excitation and emission slits and wave lengths set at 5/5 and 390/470 nm, respectively.

Free and total thiol group contents were measured colorimetrically using 2 mM Ellman's reagent [5,5′-dithio-bis (2-nitrobenzoic acid); DTNB] (Chandrapala et al., 2011). The absorbance of the yellow coloured compound was measured at 412 nm by UV spectrophotometer (Biochrom LibraS11, Cambridge, UK) and the net absorbance was calculated by the difference between samples with/without the addition of DTNB.

Surface charge and particle size distribution of whey concentrates were measured by laser-Doppler electrophoresis and dynamic light scattering, respectively, using a Zetasizer Nano ZSP (Malvern Instruments Ltd, Malvern, UK) with a Refractive Index (RI) of 1.45.

Surface charge of whey protein powders was determined by laser-Doppler electrophoresis using the same Malvern Zetasizer Nano ZSP while ethanol (100%, w/v) with RI of 1.36 was used as the dispersant. Particle size distribution of whey powders was measured as described by Nijdam and Langrish (2006) using a Malvern Master Sizer S (Malvern Instruments Ltd). Isopropanol was used as the dispersant, considering 1.45 and 1.37 as the RI of whey protein powder and isopropanol, respectively. The average of three measurements was taken and the size is expressed as D (0.5), the volume − weighted median diameter.

The Fourier transform infrared (FTIR) spectra of whey concentrates and corresponding powders were obtained in the range of 4000–600 cm^{-1} using the PerkinElmer Frontier FTIR spectrometer (PerkinElmer, MA, USA). The spectra were obtained as an average of 16 scans and recorded at a 2 cm^{-1} resolution, which were then vector normalised, smoothened and deconvoluted with the aid of IR Solution software (Shimadzu Corporation), to recognise the corresponding peaks within the amide I region of 1600–1700 cm^{-1}.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing and non-reducing conditions (Nishanthi, Vasiljevic, & Chandrapala, 2017), using freshly casted gels with 30% acrylamide and 10% SDS. Samples were reduced with addition of β-mercaptoethanol and subsequent heating at 100 °C for 5 min. For comparison purposes between bands, relative band intensity (RBI) of a particular band was calculated. The RBI was defined as:

$$RBI = \frac{Band\ intensity}{Total\ lane\ intensity} \quad (1)$$

2.4. Surface characterisation of spray dried powders

X-ray photoelectron spectroscopy (XPS) was carried out to determine the elemental composition of powders using a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) with a monochromated aluminium X-ray source (10 mA, 15 kV) within 10 nm of the surface as described by Fyfe et al. (2011). The elemental ratios were further interpreted as the amounts of fat, protein and lactose according to the method described by Faldt, Bergenstahl, and Carlsson (1993). It was assumed that elemental

composition of the pure components is a linear combination with the three major components of the sample (Faldt et al., 1993; Kim, Chen, & Pearce, 2003).

Visualisation of microstructure of four whey powders was performed using the JEOL NeoScope JCM-5000A scanning electron microscope (SEM) (Tokyo, Japan). The powder samples were mounted on aluminium stubs using a double-sided adhesive tape by spraying the powders on the tape. The samples were then coated with gold for 3 min in a Polaran SC7640 sputter coater (VG Microtech, UK) and were examined by SEM at an accelerating voltage of 10 kV under high vacuum.

2.5. Statistical analysis

The experiments were organised in a randomised blocked design with one main factor type of whey and the replications as blocks. All samples were replicated and subsampled resulting in at least 4 independent observations ($n \geq 4$). Mean of at least 4 independent observations was used for the comparison purposes.

3. Results

3.1. Composition and physical characteristics of the whey concentrates

Sweet whey, acid whey and salty whey concentrates (SWC, AWC, and StWC, respectively) produced by ultrafiltration varied in terms of composition and protein characteristics with reference to the native whey concentrate (NWC) (Tables 1 and 2). AWC was significantly higher in Ca (0.14%) and lactic acid (LA) (0.6%), while StWC was higher in Na (1.1%) as compared with other whey concentrates (Table 1). Acidification by starter culture during the Greek yoghurt manufacturing and the salting step during cheese manufacturing contributed to these compositional differences in AWC and StWC, respectively. Significantly ($p < 0.05$) higher thiol activity was observed in AWC and StWC as compared with that in NWC and SWC (Table 2). Average particle size of four whey concentrates was significantly different ($p < 0.05$), being the greatest (3327 nm) and least (271 nm) for AWC and StWC, respectively. The most and least negative surface charge was observed for NWC (−10.7 mV) and AWC (−3.5 mV), respectively. Significantly higher ($p < 0.05$) surface hydrophobicity was observed for AWC, while the surface hydrophobicity of StWC was lower (135) compared with other whey concentrates.

3.2. Composition and physical characteristics of the whey powders

Four whey powders exhibited variations in their composition and physical properties (Table 3). Surface charge of salty whey powder was positive (7.1 mV) and significantly greater ($p < 0.05$) than that of other three whey powders. Particle size of sweet whey powder was significantly ($p < 0.05$) larger (27.8 μm) compared with that of native, acid and salty whey powders.

Proteins predominated on the surfaces of all four whey powders (Table 4). Native whey powder contained almost 81% of the proteins on the surface, whereas slightly less protein coverage was observed in acid (78%) and sweet whey (70%). In contrast, surface protein coverage of salty whey powder was the lowest (40%). However, fat covered a considerable surface area of the salty whey powder particle (37%), whereas surface fat coverage lied between 10 and 12% for the other three whey powders.

The hydrophobicity of the particle surface was estimated by analysing the elemental bonding state of C in the near surface region (10 nm) of the particle (Table 4). It was presumed that the increased non-polar bonds (C—C) at the surface are associated with increased hydrophobicity of the particle surface (Fyfe et al., 2011). Salty whey was characterised with the highest proportion of C—C bonds (63.8%) on the particle surface, followed by native (51%), acid (48.9%) and sweet (48.6%) whey powders, indicating that particle surface of the salty whey powder was the most hydrophobic.

3.3. Structural characterisation of whey concentrates and powders

Secondary protein structure of WPs primarily consists of β-sheets and α-helixes, giving them their distinctive globular appearance. Therefore, the peak intensity of these and possible aggregated structures were analysed to establish structural changes induced by compositional and processing differences and is represented in Fig. 1A and B, respectively, for whey concentrates (A) and whey powders (B).

The β-sheet conformation was equally apparent in both NWC and native whey powder in the region of 1626–1638 cm^{-1} (Adochitei & Drochioiu, 2011; Kong & Yu, 2007). Several peaks depicting intermolecular β-sheet aggregation were apparent in NWC (1681 cm^{-1}, 1617 cm^{-1} and 1609 cm^{-1}) and the powder (1680 cm^{-1} and 1610 cm^{-1}) (Kong & Yu, 2007). However, the extent of β-sheet aggregation was similar in both the concentrate and powder. Furthermore, NWC showed a great peak intensity for α-helixes (1658 cm^{-1}) (Adochitei & Drochioiu, 2011; Kong & Yu, 2007), whereas the intensity of this peak diminished in the

Table 1
Compositional analysis of four whey concentrates.[a]

Component	Native whey	Sweet whey	Acid whey	Salty whey
Total solid (%)	2.5 ± 0.01[a]	16.8 ± 0.01[b]	2.86 ± 0.07[c]	8.9 ± 0.01[d]
Ash (%)	0.2 ± 0.003[a]	0.7 ± 0.08[b]	0.21 ± 0.01[a]	1.7 ± 0.11[c]
Total protein (%)	2.2 ± 0.01[a]	10.8 ± 0.4[b]	1.73 ± 0.01[c]	1.0 ± 0.1[c]
Non-protein nitrogen (%)	0.005 ± 0.001[a]	0.01 ± 0.0[a]	0.006 ± 0.001[a]	0.01 ± 0.0[a]
pH	6.5 ± 0.03[a]	6.4 ± 0.02[b]	4.2 ± 0.02[c]	5.5 ± 0.01[d]
Lactose (%)	0.1 ± 0.02[a]	2.9 ± 0.03[b]	0.7 ± 0.02[c]	2.4 ± 0.3[c]
Lactic acid (%)	0.001 ± 0.0001[a]	0.1 ± 0.03[b]	0.2 ± 0.02[c]	0.07 ± 0.001[b]
Ca (mg 100 g^{-1})	40 ± 1[a]	20 ± 3[b]	140 ± 10[c]	80 ± 1[c]
K (mg 100 g^{-1})	30 ± 1[a]	100 ± 20[b]	10 ± 0.0[a]	50 ± 6[a]
Mg (mg 100 g^{-1})	20 ± 1[a]	10 ± 1[b]	10 ± 1[b]	10 ± 6[b]
Na (mg 100 g^{-1})	10 ± 4[a]	400 ± 20[a]	30 ± 10[b]	1100 ± 50[b]
Inorganic phosphate (mg 100 g^{-1})	10 ± 6[a]	10 ± 3[a]	3 ± 1[a]	10 ± 3[a]
Total phosphate (mg 100 g^{-1})	20 ± 2[a]	30 ± 6[a]	10 ± 3[a]	20 ± 1[a]

[a] Values are means ± standard deviation (SD) of at least 4 independent measurements ($n \geq 4$); different lowercase superscript letters in the same row depict the significant difference between means for each whey concentrate.

Table 2
Physical characteristics of four whey concentrates.[a]

Parameter	Native whey	Sweet whey	Acid whey	Salty whey
Free thiol (μmol g^{-1} protein)	39.3 ± 4.9[a]	72.1 ± 4.3[b]	6.6 ± 0.25[c]	7.08 ± 0.3[c]
Total thiol (μmol g^{-1} protein)	380 ± 7[a]	781 ± 5[b]	409 ± 2[c]	354 ± 16[d]
Particle size (nm)	540 ± 12[a]	1170 ± 23[b]	3327 ± 25[c]	271 ± 1[d]
Zeta potential (mV)	−10.7 ± 0.6[a]	−7.0 ± 0.5[b]	−3.5 ± 0.3[c]	−7.2 ± 0.17[b]
Surface hydrophobicity	160 ± 10[a]	155 ± 8[a]	418 ± 3[b]	135 ± 2[c]

[a] Values means ± standard deviation (SD) of at least 4 independent measurements (n ≥ 4); different lowercase superscript letters in the same row depict the significant difference between means for each whey concentrate.

Table 3
Moisture content, total protein content, zeta potential and particle size of whey powders.[a]

Parameter	Native whey	Sweet whey	Acid whey	Salty whey
Moisture (%)	5.5 ± 0.7[ab]	4.1 ± 0.2[ac]	5.7 ± 0.8[b]	3.9 ± 0.08[c]
Total protein (%)	84.1 ± 0.6[a]	61.8 ± 0.1[b]	67 ± 0.3[c]	10.9 ± 0.2[d]
Zeta potential (mV)	−34.4 ± 2.8[a]	−30.8 ± 1.15[a]	−11.7 ± 2.2[a]	7.1 ± 0.8[b]
Particle size (μm)	22.4 ± 0.7[a]	27.8 ± 0.1[b]	22.8 ± 1.2[a]	22.9 ± 0.1[a]

[a] Values are means ± standard deviation (SD) of at least 4 independent measurements (n ≥ 4); different lowercase superscript letters in the same row depict the significant difference between means for each whey powder.

Table 4
Surface composition and elemental state of C (C–C) in four whey powders.

Sample	Surface composition of powder particles (%)				Elemental state of C (C–C) (%)
	Fat	Lactose	Protein	Mineral	
Native whey	12	6	81	0.3	51
Sweet whey	10	20	70	0.8	48.6
Acid whey	11	10	78	0.3	48.9
Salty whey	37	16	40	1.8	63.8

powder. Disruption of α-helixes was concurrent with appearance of random coils at 1643 cm^{-1} in the powder.

SWC was characterised with β-sheet configuration (1629 cm^{-1}) (Fig. 1A). Upon drying, the peak representing β-sheets broadened, implying a partial unfolding and crosslinking (1610 cm^{-1}) (Rahaman, Vasiljevic, & Ramchandran, 2015). SWC and powder showed less α-helix configuration while random coils (1643 cm^{-1}) were remarkably high.

In comparison with AWC, acid whey powder contained less concentration of β-sheet conformations. Aggregation driven by β-sheet interlinking was apparent in both samples denoted by two peaks at 1621 cm^{-1} and 1610 cm^{-1}, while an additional peak at 1680 cm^{-1} representing intermolecular β-sheet crosslinking appeared only in the powder (Lefèvre & Subirade, 1999). Compared with AWC, α-helixes at 1658 cm^{-1} largely diminished in the powder, while random coils (1645 cm^{-1}) intensified.

Peaks attributed to β-sheets were absent in StWC; although an appearance of a small peak at 1682 cm^{-1} indicating the aggregation driven by intermolecular crosslinking of β-sheet was prominent. On the contrary, salty whey powder (Fig. 1B) exhibited both the presence of β-sheets and intermolecularly aggregated β-sheets at 1632 cm^{-1} and 1682 cm^{-1}, respectively. Similar to β-sheets, α-helix peaks were missing in StWC, however reappeared in salty whey powder after spray drying.

3.4. Prevalence of protein interactions in whey concentrates and powders

SDS-PAGE patterns for whey concentrates (A, non-reduced; B, reduced) and whey powders (C, non-reduced; D, reduced) are shown in Fig. 2. Protein aggregates (≥250 kDa) present in non-reduced and reduced samples were linked with covalent-disulphide and non-disulphide bonds, respectively. Therefore, the amount of protein aggregates linked with disulphide bonds was calculated from the difference between the amount of protein aggregates present in non-reduced and reduced samples.

NWC (Fig. 2A and B) contained protein aggregates (≥250 kDa) with relative band intensity (RBI) of 0.134 in the non-reduced state, while RBI of protein aggregates after reduction was 0.031. Accordingly, 77% of protein aggregates in NWC were linked via disulphide bonds. Increased RBI of κ-casein and bovine serum albumin (BSA) in reduced samples implied that disulphide bonds were prevalent between these two proteins. Compared with NWC, the amount of protein aggregates linked via covalent bonds was 38% greater in the native whey powder (Fig. 2C and D). Following the reduction of covalently bonded aggregates present in the powder, caseins (RBI, 0.041) appeared, as they were absent in non-reduced samples, β-lactoglobulin (β-LG) concentration increased by 18% and concentration of BSA doubled, suggesting a possible existence of thiol/disulphide links between β-LG, BSA and caseins (Oldfield, Taylor, & Singh, 2005).

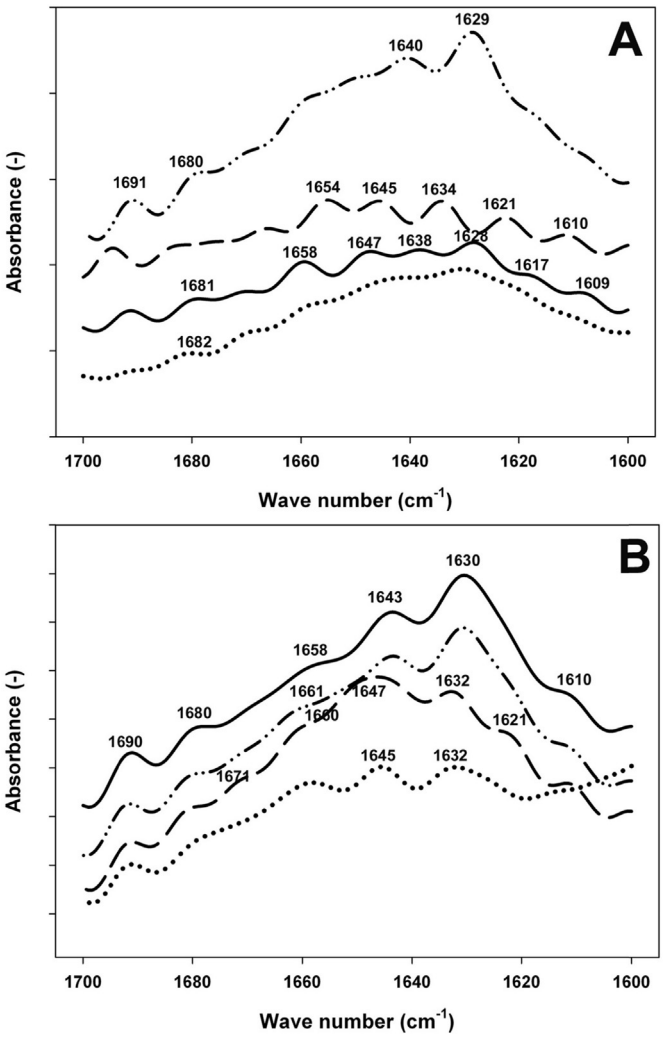

Fig. 1. Vector normalised, smoothened and deconvoluted FTIR spectra of the Amide I region (1600–1700 cm^{-1}) for whey concentrates (A) and whey powders (B) of native whey (solid line), acid whey (dashed), sweet whey (dotted and dashed line) and salty whey (dotted line).

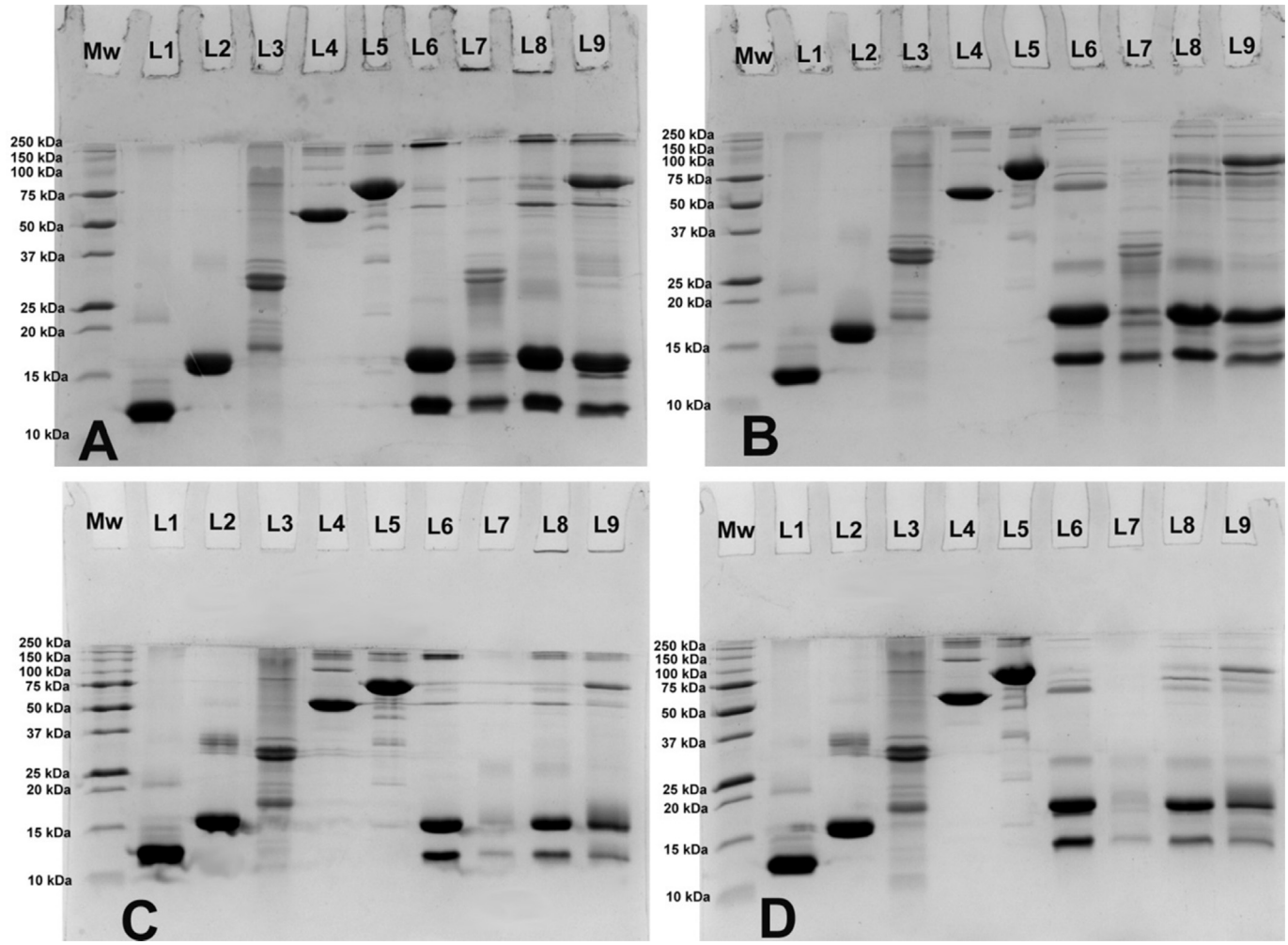

Fig. 2. Sodium dodecylsulphate-polyacrylamide gel patterns of proteins present in whey concentrates (A, non-reducing; B, reducing) and whey powders (C, non-reducing; D, reducing). Lanes as indicated as; Mw, molecular mass marker; L1, α-lactalbumin standard; L2, β-lactoglobulin standard; L3, caseins standards including αs-, β- and κ-casein; L4, bovine serum albumin standard; L5, lactoferrin standard; L6, native whey; L7, acid whey; L8, sweet whey; L9, salty whey.

SWC contained large protein aggregates (≥250 kDa) accounting for RBI of 0.134 under non-reducing conditions, which decreased to a RBI of 0.024 after reduction indicating the presence of ~82% of protein aggregates covalently linked by disulphide bonds. Increased band intensities of κ-casein and β-LG under reducing conditions indicated predominant covalent bonding between these two proteins. In sweet whey powder, protein aggregates (≥250 kDa) were present in both non-reduced (RBI, 0.046) and reduced (RBI, 0.022) samples denoting approximately equal concentration of aggregates linked via covalent-disulphide and non-disulphide bonds. Under reducing conditions, the RBI of κ-casein and β-LG increased, suggesting that disulphide links were mainly formed between these proteins in the powder (Fig. 2C).

The RBI of protein aggregates (≥250 kDa) in AWC decreased from 0.082 to 0.042 after reduction with β-mercaptoethanol, implying that AWC contains approximately equal amounts of protein aggregates formed via covalent-disulphide and non-disulphide interactions. Reduction of AWC increased the RBI of β-LG and α-lactalbumin (α-LA), suggesting possible disulphide bond between them leading into polymerisation and formation of protein aggregates. Furthermore, a fraction of these aggregates could have been formed by self-aggregation of β-LG, as shown by the intermolecular β-sheet crosslinking as determined by FTIR. In contrast, the amount of protein aggregates estimated under both non-reducing and reducing conditions was similar in acid whey powder (RBI, 0.037), suggesting that all protein aggregates present in the powder were created via covalent interactions that did not involve disulphide bonds.

Proteins aggregates (≥250 kDa) present in StWC under reducing conditions was 88% less compared with that under non-reducing conditions, implying that majority of protein aggregates was formed by covalent-disulphide bonding. The dissociation of these aggregates in StWC increased the concentration of lactoferrin (LF) implying a possible intramolecular covalent linking among LF molecules. Salty whey powder contained less disulphide-linked protein aggregates than StWC. Characteristically, StWC exhibited an intense band (RBI, 0.22) for LF, compared with other whey concentrates. Reduction of the sample did not change the band intensity, implying that this band was not covalently-linked aggregate. However, spray drying reduced the band intensity of LF by 38%.

4. Discussion

4.1. Composition and physical characteristics of the whey concentrates

The significantly high LA and Ca concentrations in AWC and the severe heat treatment applied during the Greek-yoghurt pasteurisation are responsible for the significantly high surface

hydrophobicity and the more positive surface charge as compared with other whey streams. Due to low acid dissociation constant of LA, negatively charged carboxyl groups located on the surface of WPs are protonated, leading to an overall reduction of negative charge of WPs. As negative surface charge density reduces electrostatic, van der Waals and covalent interactions prevail (Dissanayake, Ramchandran, Piyadasa, & Vasiljevic, 2013). β-LG dimers dissociate to monomers, exposing the free thiol-group (Cys_{121}) located at the outside of the β-barrel, which in turn initiates aggregation (Zhu & Damodaran, 1994). Concurrently, the disulphide bond (Cys_{66}—Cys_{160}) located on the external loop of β-LG is exposed, promoting inter-molecular thiol-disulphide exchange reactions, leading to further aggregation. This aggregation of WPs in AWC was confirmed by the significantly high particle size (3327 nm) in comparison with other whey streams.

The presence of high concentrations of Na mainly contributed to the lowest surface hydrophobicity in StWC as compared with NWC, SWC and AWC. Water molecules interact with salts favourably to mitigate the osmotic imbalance. This in turn results in protein—protein attraction via hydrophobic interactions (Hussain, Gaiani, Aberkane, & Scher, 2011), resulting in a lower surface hydrophobicity (Table 2). Interestingly, increased hydrophobic interactions in StWC had no apparent effect on the particle size (Table 2), which was the lowest as compared with other whey concentrates. In a solution with high ionic strength, a critical size of a stable nucleus is smaller; therefore, multiple nucleations occurs resulting in formation of a large number of small aggregates (Uversky & Lyubchenko, 2013).

4.2. Composition and physical characteristics of the whey powders

During spray drying, high inlet temperature and atomised distribution of the feed to the drying chamber cause spontaneous moisture evaporation from the droplets, simultaneously leading whey components to migrate towards the surface to replace the aqueous patches (Kim et al., 2003). Migration rate of whey components differs based on the surface activity, interactions state and drying temperature, thus results in a compositional gradient between the core and the crust, which ultimately affects the functional performance (Kim, Chen, & Pearce, 2009). All four whey powders had protein dominant particle surfaces (Table 4) indicating that preferential diffusion of WPs towards the surface was high in comparison with lactose and minerals (Kim et al., 2003). However, salty whey contained the least surface protein coverage suggesting restricted migration of proteins towards the surface due to preferential migration of fat towards the same surface (Faldt et al., 1993). The increased amount of surface fat led to an increased hydrophobicity on salty whey powder particles (Table 4). Denatured whey proteins are less surface active thus poorly migrate to the particle surface (Kim et al., 2009). Greater content of denatured WPs in StWC as evidenced by the lowest surface hydrophobicity and the low free and total thiol contents compared with other whey concentrates may also decrease the protein coverage on the particle surface. Compared with other whey powders, particle surface of salty whey powder contained high concentration of minerals, majorly Na, as characterised by the positive surface charge (Table 3).

According to the scanning electron microscopic (SEM) images (Fig. 3) the surface of native whey powder was wrinkled and dented with some deep and shallow folds, as typical to a protein-rich particle (Stevenson & Chen, 1996). Compared with other three powders, sweet whey powder contained largest spherical particles as evident by their particle size (27.8 μm). Formation of large, smooth surfaced particles is favoured by a high total solid content in the feed and high inlet temperature during spray drying (Kim et al., 2009). In acid whey powder, cluster-like particle agglomeration was observed attributed to the high moisture content (5.7%) imposed by a typical LA-rich environment. A dense, compact particle structure similar to a cauliflower seen in salty whey powder was due to highly hydrophobic particle surface, which promoted hydrophobic interactions with surrounding powder particles (Nijdam & Langrish, 2006). Furthermore, cluster type protein aggregation might be responsible for compact particle organisation in salty whey powder (Zhang, Arrighi, Campbell, Lonchamp, & Euston, 2016) as evident by the high thiol activity in StWC.

4.3. Structural characterisation of whey concentrates and powders

In NWC and the corresponding powder, the extent of β-sheet and its aggregated conformations were similar, implying that spray drying had no apparent effect on β-sheet unfolding or aggregation. In contrast, spray drying completely unfolded the α-helixes present in NWC, simultaneously reforming them into random coil structures. Spray drying of SWC on the other hand induced protein aggregation driven by both β-sheet crosslinking and α-helical unfolding. The greater extent of protein aggregation in sweet whey powder was supported by enlarged particle size.

Spray drying of AWC unfolded α-helixes, subsequently forming random coils. Furthermore, β-sheets in AWC participated in intermolecular crosslinking during drying. At acidic pH, destabilisation of β-LG dimer exposes free Cys_{121} residue on β-strand H, triggering intermolecular β-sheet aggregation (Kontopidis, Holt, & Sawyer, 2004) via covalent linking. Following the exposure of free Cys_{121} residue and subsequent intermolecular β-sheet aggregation, α-helix located just above the β-strand H destabilises (Kontopidis et al., 2004).

In contrast to other three whey concentrates, secondary structures were largely absent in StWC. In a highly positive ionic environment, electrostatic status of the dimer interface is disturbed, which exposes free Cys_{121} and two disulphide bonds for thiol/disulphide based reactions (Fig. 2). Spray drying of StWC resulted in appearance of native secondary structures that were previously absent in the concentrate. Perhaps the spray drying process, which removes nearly 96% of water, initiated salt crystallisation and thus minimised its osmotic influence on WPs. Absence of dissolved ionic forms of salts facilitates WPs to reach its negative repulsion, hence, reversing the aggregation and resulting in both native monomers and intermediate structures.

4.4. Prevalence of protein interactions in whey concentrates and powders

Even with a minimal processing applied in manufacturing of NWC, disulphide bonds were established between κ-casein and BSA leading to formation of protein aggregates. Spray drying, in turn increased number of these aggregates, involving β-LG in addition to BSA and caseins. At or above 70 °C, thiol-disulphide interchange reactions take place between β-LG and κ-casein. Denatured β-LG may also interact with BSA and form disulphide links leading to polymerisation. The FTIR spectrum for native whey powder showed a distortion of α-helixes and intermolecular β-sheet aggregation, which may correlate to the formation of these aggregates on the expense of native proteins.

In SWC and the corresponding powder, protein polymerisation via disulphide interactions predominantly occurred between κ-casein and β-LG. Spray drying of SWC resulted in a high number of protein aggregates linked via non-disulphide covalent links indicating formation of early Maillard reaction products created likely due to high inlet temperature used during spray drying (Bernard, Regnault, Gendreau, Charbonneau, & Relkin, 2011). As

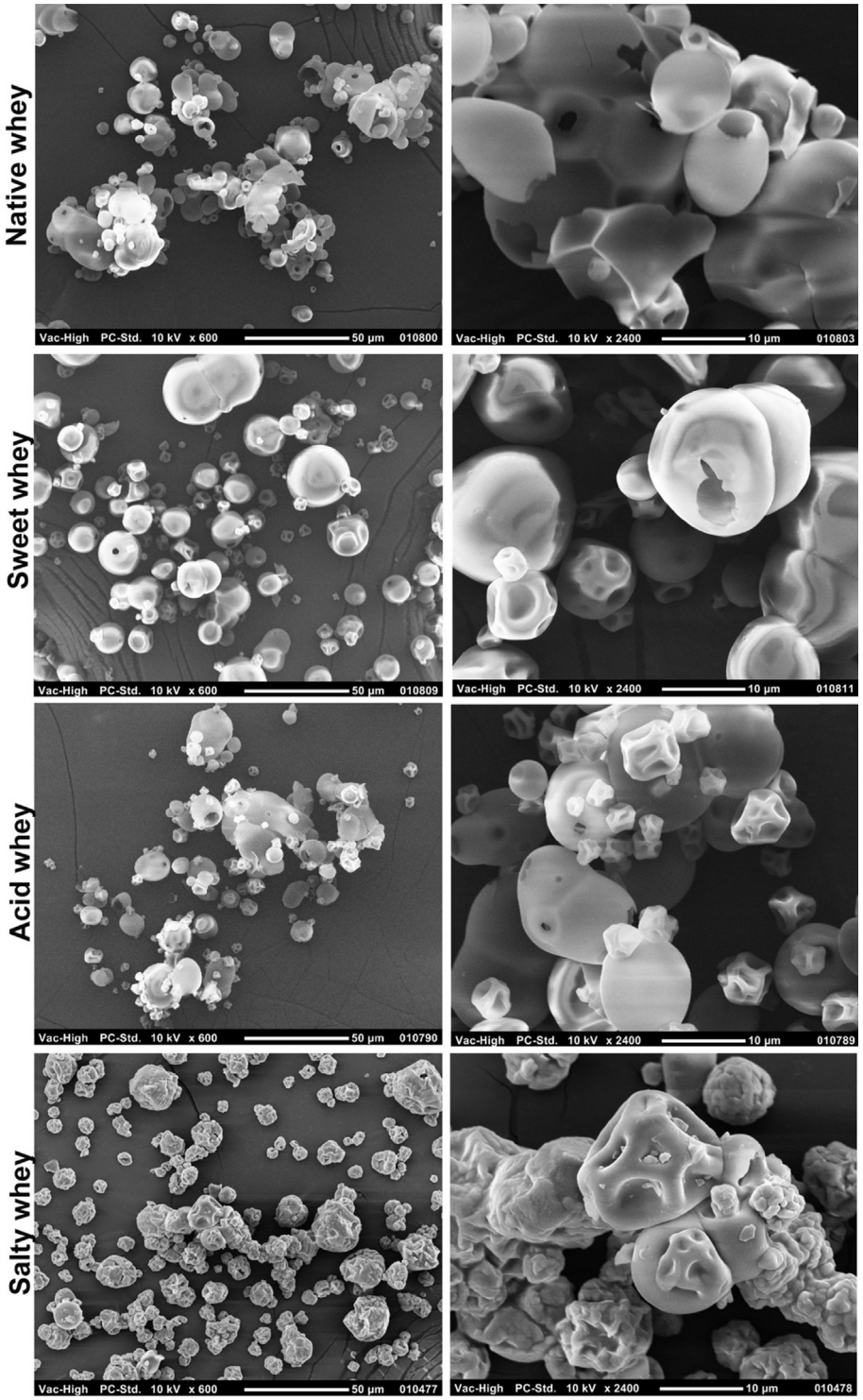

Fig. 3. Scanning electron micrographs of four whey powders at 600× and 2400× magnification.

SWC contained high lactose concentration (2.9%), spray drying likely induced lactosylation of lysine residues of β-LG and α-LA.

In contrast to NWC and SWC, AWC contained equal concentrations of protein aggregates formed via covalent-disulphide and non-disulphide bonds. Disulphide bonds mainly occur between β-LG and α-LA, while a fraction of these aggregates could have been formed by self-aggregation of β-LG, as shown by the intermolecular β-sheet crosslinking as determined by FTIR. Protein aggregation via covalent-non-disulphide bonds occurred as a result of lactosylation of proteins, forming early Maillard reaction products in response to severe pasteurisation step during Greek yoghurt manufacturing. In contrast to AWC, approximately all protein aggregates in acid whey powder were formed through covalent-non-disulphide interactions. Spray drying of acid whey powder further lactosylate

the proteins into Maillard products. FTIR data for this powder suggested a pronounced α-helix disruption, possibly reflecting creation of these unreducible aggregates.

In contrast to other three whey concentrates, the majority of protein aggregates present in StWC was formed via intramolecular covalent linking among LF molecules. Conversion process of StWC to powder reduced the amount of disulphide-linked protein aggregates. It appeared that loss of moisture during spray drying reduced the osmotic influence of salt on WPs, allowing the WPs to regain their native structure, therefore lowering the amount of WPs involved in aggregation. This was in line with the FTIR data, which showed appearance of native secondary structures in the powder, otherwise absent in the concentrate.

5. Conclusions

Changes in the properties of WPs during processing are clearly governed by the type of the whey stream. AWC and StWC were characterised with aggregated proteins, created by covalent crosslinking among individual WPs (β-LG, α-LA, BSA and LF) and/or caseins, which transpired mainly in response to compositional differences (high acidity and salinity) and heat treatments such as pasteurisation and evaporation applied during the manufacturing processes. During spray drying of NWC and SWC, protein aggregation and related physical characteristics were conveyed from the concentrates to the powders. However, transfer of protein characteristics is less likely to occur in acid and salty whey streams due to the characteristic changes associated with spray drying of AWC and StWC. During spray drying of AWC, Maillard reaction products are formed by crosslinking and polymerisation of WPs. In the absence of moisture in salty whey powder, Na salt crystallised, reversing the covalent bonding between LF molecules. Therefore, it can be concluded that the compositional, surface and particle-organisation characteristics of whey powders present a cumulative effect of those properties of original feed stream, concentrate, and changes induced by the spray drying. Fundamental knowledge generated in this study may facilitate the re-utilisation of acid and salty whey streams through composition-adjusted ultrafiltration and spray drying to manufacture whey powders that could be potentially used in various food systems.

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Chapter 5: Properties of whey protein concentrate powders obtained by spray drying of sweet, salty and acid whey under varying storage conditions

Chapter 5 reports the conformational and surface interactional changes of whey proteins present in sweet, acid and salty- whey protein concentrates under three different storage temperatures (4, 25 and 45 °C) and two relative humidity levels (22% and 33%) over a storage period of 90 days.

The paper entitles “Properties of whey protein concentrate powders obtained by spray drying of sweet, salty and acid whey under varying storage conditions” by Manjula Nishanthi, Jayani Chandrapala and Todor Vasiljevic has been published in the peer-reviewed journal “Journal of Food Engineering” (2017), 214: 137 – 146. http://dx.doi.org/10.1016/j.jfoodeng.2017.06.032

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: Manjula Nishanthi Kottahachchi Kankanamge

Signature: [redacted]

Date: 08.09.2017

Paper Title:

Properties of whey protein concentrate powders obtained by spray drying of sweet, salty and acid whey under varying storage conditions

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Manjula Kankanamge	80	Concept development, research question, hypothesis and manuscript preparation
Dr. Jayani Chandrapala	10	Concept development, research question, hypothesis and contribute in writing manuscript
Prof. Todor Vasiljevic	10	Concept development, statistical analysis, artwork preparation, contribute in writing manuscript and submission to journal

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

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2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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Properties of whey protein concentrate powders obtained by spray drying of sweet, salty and acid whey under varying storage conditions

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ABSTRACT

Changes of secondary structure and protein interactions of whey protein (WPs) present in native, sweet, acid and salty-WPCs were analyzed following storage at 4, 25 or 45 °C and 22 or 33% relative humidity for a period of 90-days. WPs aggregated predominantly through covalent crosslinking, achieving maximum at 45 °C and 33% RH. Greater participation of β-lactoglobulin in covalent crosslinking was evident in all WPCs, while that of α-lactalbumin was significantly ($p < 0.05$) high in acid-WPC only. Reaction order of β-LG denaturation in acid and salty-WPCs was approximately 2 while approximately 1 in native and sweet-WPCs. Activation energy was significantly ($p < 0.05$) higher in native and sweet-WPCs, with averages recorded as 97 and 49.8 kJ mol^{-1}, respectively, than that in acid and salty-WPCs with averages of 27.5 and 33.8 kJ mol^{-1}, respectively, mainly attributed to inherently high concentrations of lactic acid and salts in these WPCs.

1. Introduction

Whey protein concentrates (WPCs) are an important protein source used in many food systems due to their excellent functional characteristics such as emulsification, gelling and foaming. Dairy industry has been successful in utilizing the sweet whey waste stream for manufacturing of various WPCs. However, the industry has not been very successful in utilizing acid and salty whey in an equivalent way to sweet whey, mainly due to their composition-induced processing obstacles such as high acidity and salinity, respectively. Moreover, the powders produced from these two streams form clumps and sticky deposits during storage. A recent study by Nishanthi et al. (2017a) showed that the particle surface of salty-WPC was hydrophobic mainly due to high surface fat content, while that of acid-WPC was characterized by both hydrophobic and hydrophilic nature due to presence of elevated protein concentration. Therefore, these two WPCs possess a high potential to be used as functional ingredients in food systems. Thus, a thorough fundamental understanding on the stability of proteins present in these respective WPCs as affected by storage is a necessity in evaluating the potential of using these two particular WPCs.

Storage conditions induce changes in protein conformation at a molecular level, which in turn may affect functional properties of the protein. Intermolecular covalent and non-covalent interactions occur between whey proteins (WPs), resulting in protein unfolding and denaturation, eventually leading to reversible and irreversible aggregation. As a result, a proportion of native WPs decreases after 3 months of storage at 40 °C with a concomitant increase in proportion of denatured proteins. These changes were clearly temperature dependent as aforementioned changes were not noticed at 4 °C or 20 °C up to 15 months of storage (Norwood et al., 2016). At elevated storage temperatures, flexibility and mobility of WPs increases, resulting in partial loss of their tertiary structure (Norwood et al., 2016). WPC was found to have increased surface hydrophobicity and less exposed amino acids on the particle surfaces after storage at 60 °C and 22% RH for one month (Burgain et al., 2016). In addition to denaturation, WPs can be subjected to lactosylation during storage. While studies on lactosylation of WPC powders are lacking, Le et al. (2012) reported that six out of twelve lysine residues of α-lactalbumin (α-LA) would lactosylate during storage of milk protein concentrates up to 12 weeks at 40 °C and 84% RH, which indicates that similar changes could be expected in WPC, especially those containing greater lactose content. These structural modifications in WPs could subsequently affect their functional properties. For instance, formation of insoluble aggregates in response to elevated storage temperatures reduced the solubility of

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WPC (Zhou et al., 2008) affecting several functional characteristics such as emulsification, gelation and foaming.

So far, the studies mainly focused on WPC produced from the sweet whey and no study thus far has established physico-chemical properties of WPs during storage of WPCs produced from acid or salty whey, which were identified as potentially effective functional ingredients (Nishanthi et al., 2017a). Therefore, the main aim of the current study was to establish conformational and surface interactional changes of WPs present in sweet, acid and salty WPCs under three different storage temperatures (4, 25 and 45 °C) and two relative humidities (RH) (22% and 33%) over a storage period of 90 days. Native-WPC was used as the reference sample.

2. Methodology

2.1. Sample preparation

Four WPCs, native-WPC, sweet-WPC, acid-WPC and salty-WPC were manufactured in our previous study (Nishanthi et al., 2017a) with the composition shown in Table 1 and used during storage trial in the current study. WPCs were first completely dehydrated by placing appropriate quantity of samples in vacuum desiccators over phosphorous pentoxide at room temperature (25 °C) for 4 weeks.

2.2. Storage trial

Approximately 3 g of each WPC was placed in standard desiccators (with a porcelain disc size of 250 mm) with a controlled RH (22 and 33%), sealed and placed in temperature controlled cabinets at three temperatures (4, 25 and 45 °C). The equilibrium RH in each desiccator was maintained using saturated salt slurries containing potassium acetate (22± 2%) or magnesium chloride (33± 1%). At 4, 25 and 45 °C, magnesium chloride maintained RH of 34, 33 and 32%, while potassium acetate maintained RH of 24, 23 and 23%, respectively. Five desiccators were prepared for each temperature/humidity combination as sampling was conducted at 5 time points representing 0, 14, 30, 60 and 90 days of storage. A pre-trial test was conducted to estimate the equilibrium time and identify the first sampling point. Several samples from each WPC were stored in standard desiccators with saturated salt slurries of magnesium chloride and potassium acetate. After one week of storage, one sample from each WPC was tested for the water activity using a water activity meter (Aqualab CX-3T, Labcell, Hampshire, United Kingdom) at 25 °C each day. The four types of WPCs reach to the water activity of 0.22 and 0.33 by one week and four days. A stabilised water activity level could be achieved within two weeks. Therefore, the first sampling (0 day) was conducted after allowing 2-weeks equilibrium period from the initiation date of the storage trial. After opening desiccators at a required time point, the analysis was carried out within 3 days.

2.3. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of WPCs were obtained in the range of 4000–600 cm^{-1} using the PerkinElmer Frontier FTIR spectrometer (PerkinElmer, MA, USA) as described previously (Nishanthi et al., 2017b). The spectra were vector normalized, smoothened and deconvoluted with the aid of IR Solution software (Shimadzu Corporation, Kyoto, Japan), in order to recognize the corresponding peaks within the amide I region between 1600 and 1700 cm^{-1}.

2.4. Sodium Dodecyl Sulphate gel electrophoresis (SDS − PAGE)

In order to establish non-covalent and covalent interactions, Sodium Dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed under reducing (SDS^R) and non-reducing (SDS^{NR}) conditions using freshly casted gels with 30% (w/v) acrylamide and 10% (w/v) SDS (Nishanthi et al., 2017b). Samples were reduced with addition of β-mercaptoethanol and subsequent heating at 100 °C for 5 min.

2.5. Kinetics analysis

The rate (1) and the Arrhenius equation (2) were used to calculate the kinetic parameters involved in disappearance of native WPs during storage of WPCs (Oldfield et al., 2005).

$$-\frac{dC}{dt} = k_n C^n \quad (1)$$

Where:

C = Protein concentration (g kg^{-1})
t = time (days)
k_n = rate constant ((g kg^{-1})$^{(1-n)}$ day^{-1})
n = reaction order

$$k_n = k_0\, e^{\frac{-E_a}{RT}} \quad (2)$$

Where:

k_0 = pre-exponential term ((g kg^{-1})$^{(1-n)}$ day^{-1})
E_a = activation energy (kJ mol^{-1})
R = Universal Gas Constant (8.314 J mol^{-1} K^{-1})
T = temperature (K)

2.6. Statistical analysis

The experiments were organized in a randomized split plot blocked design with 3 main factors: a type of WPC as the main plot, and temperature and RH as subplots. The replications served as blocks. Replicated samples were sub sampled and analyzed. The results of the kinetic and compositional analysis were statistically

Table 1
Composition of four WPCs.

	Native-WPC	Sweet-WPC	Acid-WPC	Salty-WPC
Moisture (%)	5.5 ± 0.7^{ab}	4.1 ± 0.2^{ac}	5.7 ± 0.8^{b}	3.9 ± 0.08^{c}
Total protein (%)	84.1 ± 0.6^{a}	61.8 ± 0.1^{b}	67 ± 0.3^{c}	10.9 ± 0.2^{d}
Lactose (%)	0.1 ± 0.02^{a}	2.9 ± 0.03^{b}	0.7 ± 0.02^{c}	2.4 ± 0.3^{c}
Lactic acid (%)	0.001 ± 0.0001a	0.1 ± 0.03^{b}	0.2 ± 0.02^{c}	0.07 ± 0.001^{b}
Ca (%)	0.04 ± 0.001^{a}	0.02 ± 0.003^{b}	0.14 ± 0.01^{c}	0.08 ± 0.001^{c}
Na (%)	0.01 ± 0.004^{a}	0.4 ± 0.02^{a}	0.03 ± 0.01^{b}	1.1 ± 0.05^{b}
pH	6.5 ± 0.03^{a}	6.4 ± 0.02^{b}	4.2 ± 0.02^{c}	5.5 ± 0.01^{d}

Values are means of at least 4 independent measurements (n ≥ 4); the results are presented as means ± standard deviation (SD). Different lowercase superscripts in the same row depict the significant difference between means for each spray dried whey powder.

analyzed using the General Linear Model (GLM) and ANOVA procedure, respectively. The level of significance was preset at $p < 0.05$.

3. Results and discussion

3.1. Secondary structural changes during storage through FTIR

Significant and complex intermolecular interactions occur among WPs in response to storage conditions and associated compositional changes of examined WPCs. These changes affect the conformation of secondary structure motifs such as α-helices, β-sheets, β-turns and random coils in these proteins as shown in Figs. 1–4.

At the beginning of the storage trial, WPs present in native-WPC were characterized as β-sheet rich proteins due to two prominent peaks observed in the regions of 1628–1629 cm^{-1} and 1693 cm^{-1} (Fig. 1). However, other secondary structures, such as α-helixes, random peptides and β-turn were largely missing at 4 °C, but appeared with low intensities at 25 and 45 °C, irrespective of RH. In addition to the native moieties, β-sheet cross linking (1682 cm^{-1} and 1615 cm^{-1}) observed at the beginning of storage can be attributed to the thermal impacts associated with pasteurization and spray drying applied during processing of this WPC. During initial stages of the storage (14–30 days), secondary conformations did not change substantially. However, after 60 days of storage WPs underwent several major structural changes. These changes were more pronounced at 33% RH as compared to that at 22% RH, but were independent of storage temperature. Firstly, complete distortion of α-helixes occurred under all storage conditions, which in turn increased the intensity of randomly arranged peptides (1644 cm^{-1}). In presence of high amount of moisture (33% RH), hydrophilic amino acid residues of α-helix form hydrogen bonds with water molecules, thus disturbing its intermolecular interactions. As a result of α-helical stretching, intensity of β-turns (1660 cm^{-1}) increased attributed to the broadening of β-turn between β-strand I and H in β-lactoglobulin (β-LG). Secondly, widening of peaks attributed to β-sheets, observed prominently at 33% RH, suggests a possible intermolecular cross linking in the presence of elevated moisture content. Concomitantly, intensity of anti-parallel β-sheets (Lefèvre and Subirade, 1999) (1691 cm^{-1}) increased, suggesting a dimerization or polymerization, moving high number of β-sheets to the molecular interior. Thirdly, greater exposure of amino acid (AA) side chain residues (1610 cm^{-1}) (Lefèvre and Subirade, 1999) were observed mainly at 33% RH. The random coils formed by stretching α-helixes may be small with high exposed surface area, which subsequently expose a high number of AA residues. Moreover, protein hydration due to greater relative humidity leads to movement of hydrophilic AA residues to the molecular exterior. At the end of the storage (90 days), the peaks representing β-turns and random coils widened and disappeared suggesting a possible aggregation that destructed native monomeric and dimeric forms of proteins. This loss of secondary structures was especially magnified in the samples stored at 45 °C and 33% RH. Further, the exposed side chain residues disappeared under all storage conditions. Interestingly, a greater content of β-sheets remained unaffected, suggesting the preservation of the calyx of β-LG.

Sweet-WPC mainly contained intramolecular β-sheets (1630 and 1693 cm^{-1}) and intermolecular β-sheets (1615 cm^{-1}) at the start of the storage (Fig. 2). Interlinking of β-sheets observed at the beginning of the storage suggests an impact of thermal treatments associated with the manufacturing process of sweet-WPC (Nishanthi et al., 2017a). Similar to native-WPC, initial storage period (14–30 days) did not change the profile of the secondary structure of sweet-WPC to a great extent for most storage conditions, although random coils, α-helixes and β-turns appeared in the samples stored at 33% RH. These changes can be attributed to the protein hydration, leading acquisition of some native attributes. Furthermore, a greater exposure of side chain AA residues was observed (1611 cm^{-1}) at 22% RH. Similar to native-WPC, substantial structural changes were noticed after 60 days of storage. Irrespective of storage conditions, β-sheets lost their conformational

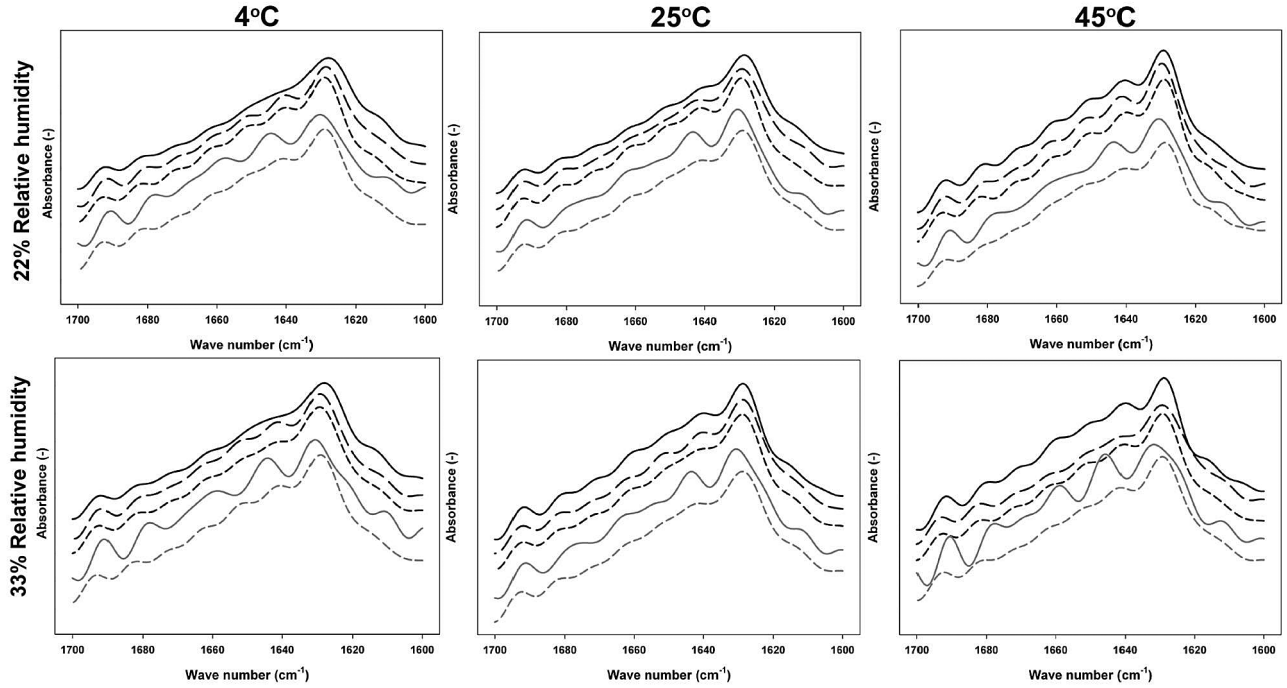

Fig. 1. Vector normalized, smoothened and deconvoluted FTIR spectra of the Amide I region (1600–1700 cm^{-1}) for native-WPC over storage period of 0 (solid-black), 14 (long dashed-black), 30 (short dashed-black), 60 (solid-grey) and 90 day (dashed-grey) under different storage temperatures (4, 25 and 45 °C) and RH (22 and 33%).

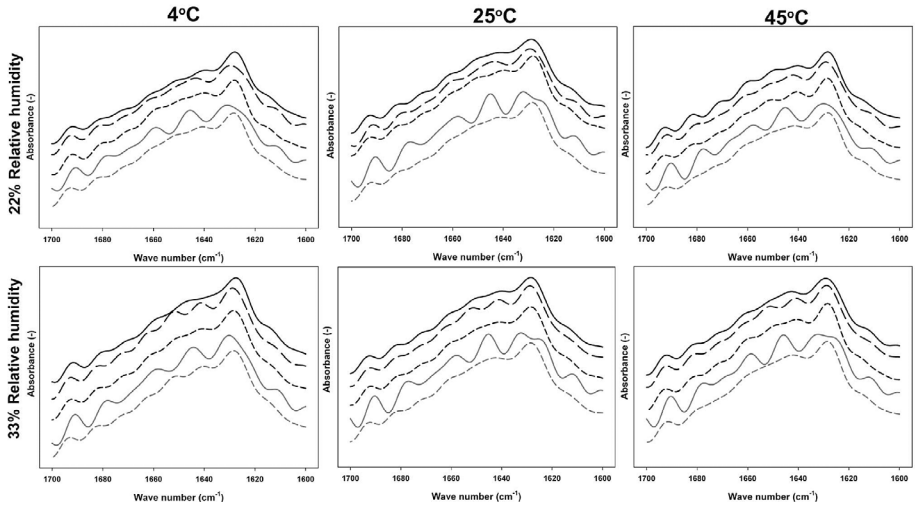

Fig. 2. Vector normalized, smoothened and deconvoluted FTIR spectra of the Amide I region (1600–1700 cm^{-1}) for sweet-WPC over storage period of 0 (solid-black), 14 (long dashed-black), 30 (short dashed-black), 60 (solid-grey) and 90 day (dashed-grey) under different storage temperatures (4, 25 and 45 °C) and RH (22 and 33%).

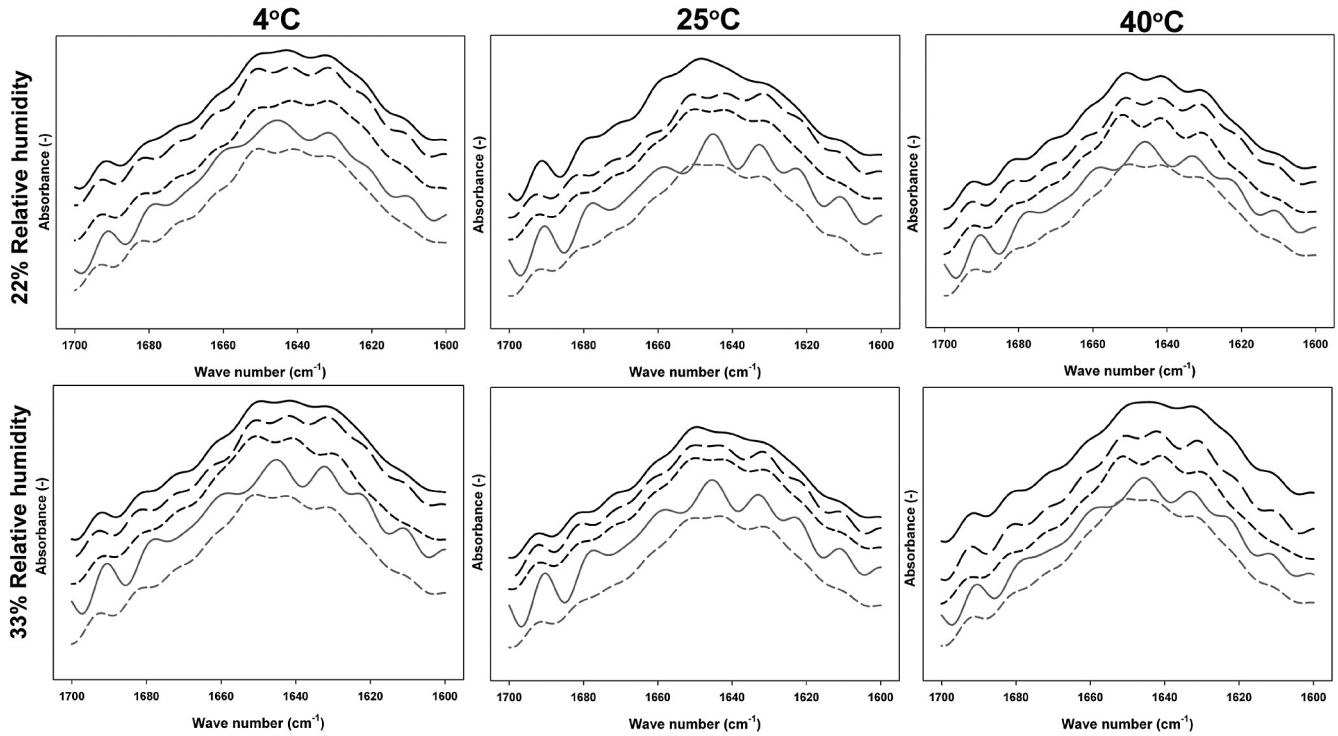

Fig. 3. Vector normalized, smoothened and deconvoluted FTIR spectra of the Amide I region (1600–1700 cm^{-1}) for acid-WPC over storage period of 0 (solid-black), 14 (long dashed-black), 30 (short dashed-black), 60 (solid-grey) and 90 day (dashed-grey) under different storage temperatures (4, 25 and 45 °C) and RH (22 and 33%).

stability, partially forming cross linked β-sheets (1680 cm^{-1}). Strong modifications were seen in β-sheets indicated by appearance of two peaks (1623 and 1634 cm^{-1}), which can be attributed to the disruption of intramolecular hydrogen bonds, leading to formation of new stronger intermolecular hydrogen bonds (Ngarize et al., 2004). This modification was especially prominent at 25 and 45 °C. Monomer-dimer equilibrium of β-LG molecules (Lefèvre and Subirade, 1999) has shifted towards the dimeric form, resulting in strongly bonded β-strand structures. Dimer formation, in parallel, leads to a high number of deeply buried β-type structures (1691 cm^{-1}). Moreover, dimer formation takes place at expense of intramolecular α-helix, subsequently, resulting in high number of random coils. The α-helix stretching into random coils was high at 25 and 45 °C, while independent of RH. Therefore, the dimerization which was established in sweet-WPC after 60 days was a result of storage temperature. By the end of storage, secondary structures other than β-sheets completely disappeared in the samples stored at 25 and 45 °C. Furthermore, aging of sweet-WPC after 60 days induced a conformational change to the calyx of β-LG indicated by a shift in peak at 1691 cm^{-1} to a peak with low intensity at 1694 cm^{-1}. This change in turn may lead to the exposure of reactive sites for covalent and hydrophobic interactions.

In acid-WPC, β-sheets (1620–1635 cm^{-1}) were largely absent at the beginning of the storage (Fig. 3). Simultaneously, aggregation

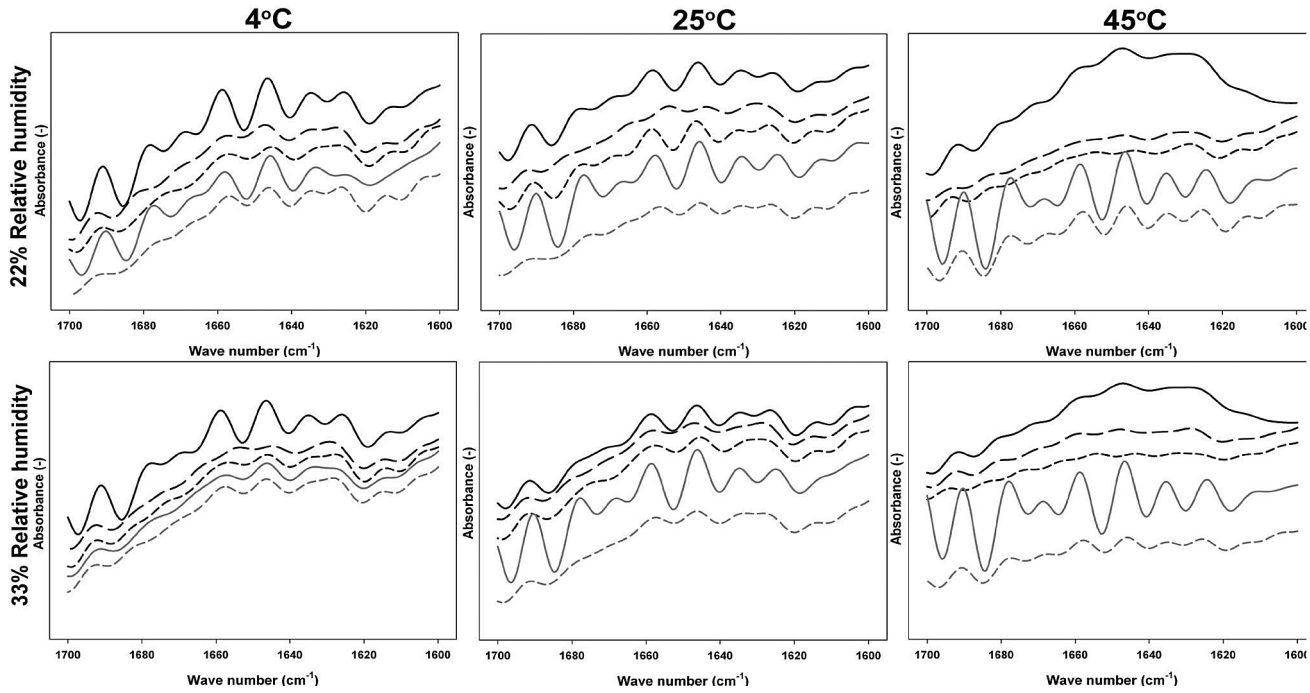

Fig. 4. Vector normalized, smoothened and deconvoluted FTIR spectra of the Amide I region (1600–1700 cm^{-1}) for salty-WPC over storage period of 0 (solid-black), 14 (long dashed-black), 30 (short dashed-black), 60 (solid-grey) and 90 day (dashed-grey) under different storage temperatures (4, 25 and 45 °C) and RH (22 and 33%).

between anti-parallel β-sheets (1682 cm^{-1}) was prominent at 22% RH and 45 °C, involving mainly β-strands located on the surface, as opposed to those in deep areas of β-LG (1691 cm^{-1}), which remained intact. Other secondary structures, such as α-helixes, β-turns and random coils were present at a low intensity, regardless of the storage conditions. By 14 days of storage, additional exposure of β-sheets (1621 cm^{-1}) occurred suggesting a partial unfolding of β-LG in response to lactic acid (LA)-induced WP alterations (Nishanthi et al., 2017b). Unfolding of β-sheets was more prominent at 33% RH as compared to that at 22% RH. Deprotonation of LA occurs rapidly in the presence of moisture due to its low acid dissociation constant (pK_a), which in turn decreases the negative surface charge density of the proteins promoting attractive forces, inducing protein aggregation driven by van der Waals and electrostatic interactions (Israelachvili, 2011). After 60 days of storage, three significant modifications were noticed. Firstly, α-helixes disappeared completely forming non-native random structures under all storage conditions. Secondly, conformation of β-sheets changed substantially, converting into distinctive two peaks in the region of 1620–1638 cm^{-1}, implying a dimerization of β-LG. In addition, under all storage conditions the intensity of deeply located β-sheets (1693 cm^{-1}) was doubled. In presence of LA, β-LG formed dimers, relocating more of β-sheets to the inner side of the molecule. Thirdly, side chain amino acid residues (1610 cm^{-1}) were greatly exposed, especially at 22% RH as a result of β-sheet modifications and α-helix disruption. Towards the end of the storage (90 day), a greater loss of secondary structures was observed, especially in the samples stored at 25 and 45 °C under 22% RH. A greater loss of random coils and dimeric forms of β-LG suggested a severe aggregation.

In contrast to other WPCs, secondary structures of salty-WPC (Fig. 4) showed high sensitivity to storage conditions during the first days of storage. For instance, proportions of β-sheets (1626, 1636 and 1693 cm^{-1}), random peptides (1648 cm^{-1}) and β-turns (1660 cm^{-1}) clearly declined as temperature and humidity increased. In parallel, a number of deeply located β-sheets (1693 cm^{-1}) descended with increase in temperature, denoting an exposure of the β-LG core. However, the temperature-dependent reactivity of β-LG did not increase the proportion of cross linked β-sheets (1616 and 1680 cm^{-1}). Due to osmotic pressure caused by Na^+ in the medium, β-sheets did not interlink, instead unfolded, forming unordered structures, as apparent by intensity of high random coil. A dimeric form of β-LG was also observed at 4 and 25 °C (1620–1636 cm^{-1}), as opposed to monomeric β-LG at 45 °C. Other secondary motifs were also greatly absent at 45 °C. During further storage (14–30 days), monomeric β-LG dominated (Lefèvre and Subirade, 1999) under all storage conditions, suggesting that native-dimers dissociated with aging. With increase in temperature, concomitant reduction of non-native monomers and enhanced aggregation driven by crosslinking of β-sheets (1616 cm^{-1}) occurred due to temperature-dependent exposure of reactive sites in β-LG. After 60 days of storage, β-sheets reappeared in the region of 1620–1638 cm^{-1}, whereas the peak intensity increased with the storage temperature. Interestingly, the region denoted β-sheets (1620–1636 cm^{-1}) consisted of two peaks at 25 and 45 °C, denoting dimeric forms of β-LG, while it was just a single peak at 4 °C, attributed to monomeric β-LG. Dimeric β-LG formation in turn increased the number of intermolecular β-sheets (1680 cm^{-1}), resulting in substantially greater proportion of deeply located β-sheets (1691 cm^{-1}) and high number of random coils and β-turns. Up to 60 days, the observed structural changes were independent of the humidity. However, towards the end of storage (90 day), humidity influenced the interactions among β-sheets. High occurrence of intermolecular hydrogen bonds prominent at 22% RH suggested a high occurrence of β-LG dimers (1620–1636 cm^{-1}), while intramolecular hydrogen bonds predominated at 33% RH, suggesting more of monomeric form of β-LG. The prolonged exposure to high humidity reduced the protein-protein interactions, instead allowing protein hydration and formation of near-native protein monomers. This assumption is also supported by the occurrence of less cross linked β-sheets (1615 cm^{-1}) at 33% RH.

3.2. Changes in protein interactions and aggregation during storage

At the beginning of the storage trial, aggregates (Mw ≥ 250 kDa) covalently linked via disulphide bridges were observed in native-WPC (Figs. 5 and 6) accompanied with subsequent disappearance of β-LG directly related to temperature, humidity and storage time. Intensity of α-LA bands on the other hand remained approximately consistent during initial 30 days of storage, but started to decline after this period implying that α-LA would be involved in the overall aggregation process at a later stage. It is known that α-LA contains only four disulphide bonds positioned at Cys_6-Cys_{120}, Cys_{28}-Cys_{11}, Cys_{61}-Cys_{77} and Cys_{73}-Cys_{91} (McSweeney and Fox, 2013), but no free thiol groups. In addition, the holo-form of α-LA, which is present abundantly in native-WPC, provides a high thermal stability (Boye and Alli, 2000). For these reasons, involvement of α-LA in overall aggregation is limited. Small amount of caseins was present in this WPC, which appeared to be involved in disulphide linking over the storage period, with the greatest extent at 45 °C and 33% RH. Thiol/disulphide exchange reaction usually occurs between Cys_{160} of β-LG and free thiols of Cys_{11} and Cys_{88} of κ-casein (Creamer et al., 2004) forming an aggregate with a minimum Mw of ~37 kDa (Fig. 5). Similar to other proteins, bovine serum albumin (BSA) was also involved in aggregation. However, its involvement was clearly storage time dependent, while other main factors had no effect. BSA forms disulphide crosslinks with β-LG (Matsudomi et al., 1994) due to a free thiol group at Cys_{34}. In addition to disulphide bridging, WPs in native-WPC participated in non-reducible aggregation (Fig. 7), likely due to lactosylation of lysine residues in these proteins. Compared to other WPCs, lactosylation was less prominent in this WPC without a clear association with storage temperature and time, which could have been caused by a very low amount of lactose.

Sweet-WPC samples exhibited a similar behavior to that of native-WPC presented by clear formation of protein aggregates covalently linked by disulphide bridging over the overall storage period (Figs. 5 and 6), again achieving maximum aggregation at 45 °C and 33% RH. In parallel to the aggregate formation, a rate of β-LG disappearance was the greatest under the same conditions (Table 2), suggesting that β-LG was the main protein involved in aggregation. Conversely, only minor involvement of α-LA in aggregation was observed under all storage conditions. No involvement of BSA was evident in sweet-WPC under all storage conditions, although comparatively a higher concentration of BSA was present in comparison with other WPCs. In contrast, caseins were involved in disulphide crosslinking, but a clear association with storage conditions and time could not be established. In addition to aggregates linked by disulphide bonds, two distinct bands representing non-disulphide linked aggregates appeared with approximate Mw of 71–72 kDa and ≥250 kDa (Fig. 6). Compared to native-WPC, these aggregates were prominent in sweet-WPC, showing a gradual increase with rise in the storage temperature, humidity and time. Sweet-WPC contained 2.9% of lactose, which might have led to lactosylation of lysine segments of β-LG. Among these, Lys_8 located at N terminal, Lys_{141} located at C terminal end and Lys_{138} located in 3 turn α-helix are easily accessible, therefore, likely the primary sites of the reaction.

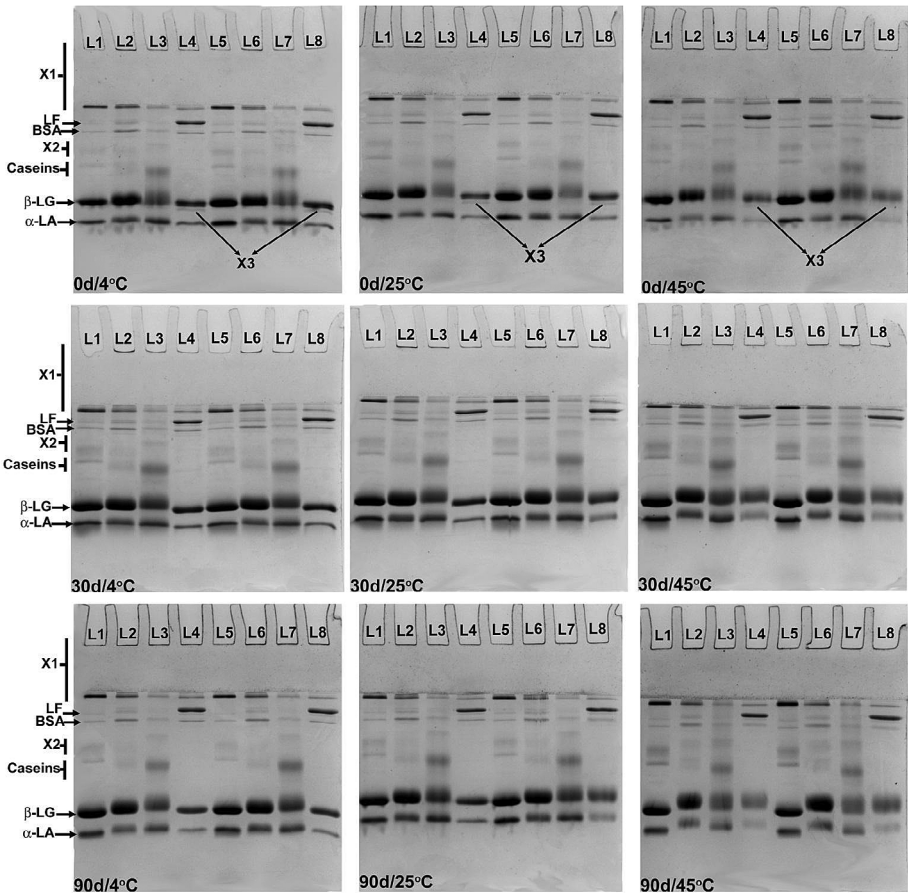

Fig. 5. SDS Non-reduced PAGE patterns of whey protein concentrate powders at 0, 30 and 90 days of storage. L1, L2, L3, L4 represent native, sweet, acid and salty-WPC under 22% RH, while L5, L6, L7, L8 represent native, sweet, acid and salty-WPC under 33% RH, respectively. X1 represent covalently linked aggregates with Mw ≥ 250 kDa. X2 represents medium size protein aggregates with Mw~35–67 kDa. X3 represent β-LG A.

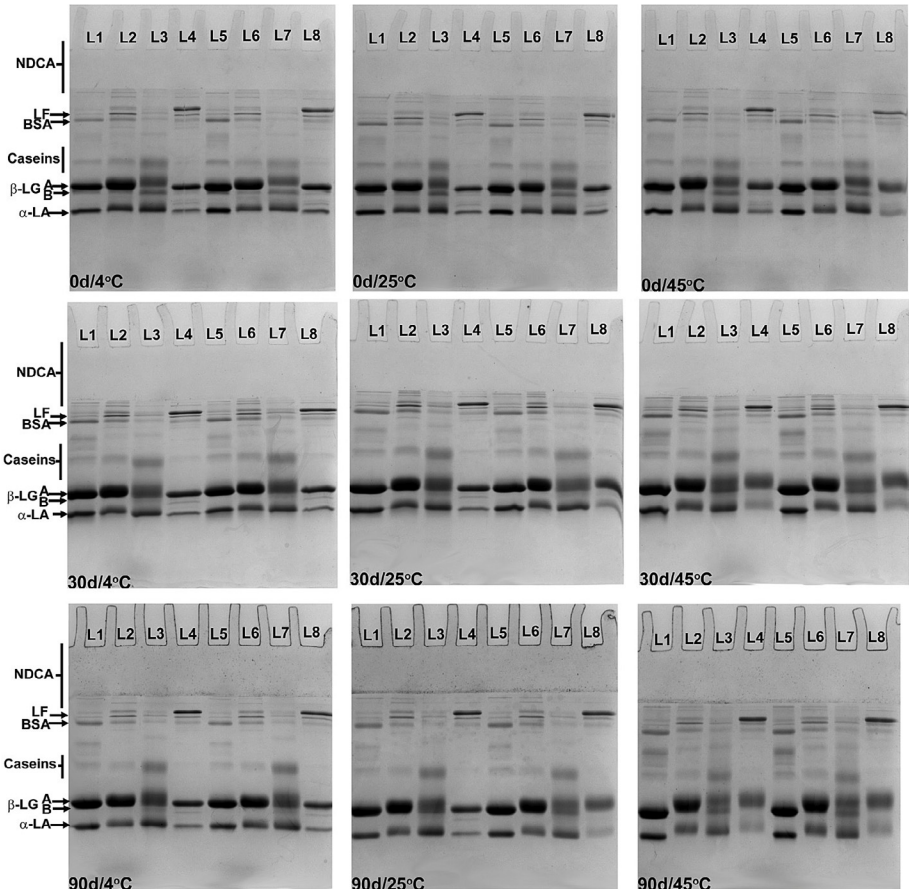

Fig. 6. SDS Reduced PAGE patterns of whey protein concentrate powders at 0, 30 and 90 days of storage. L1, L2, L3, L4 represent native, sweet, acid and salty-WPC under 22% RH, while L5, L6, L7, L8 represent native, sweet, acid and salty-WPC under 33% RH, respectively. NDCA represent non-disulphide covalently linked aggregates.

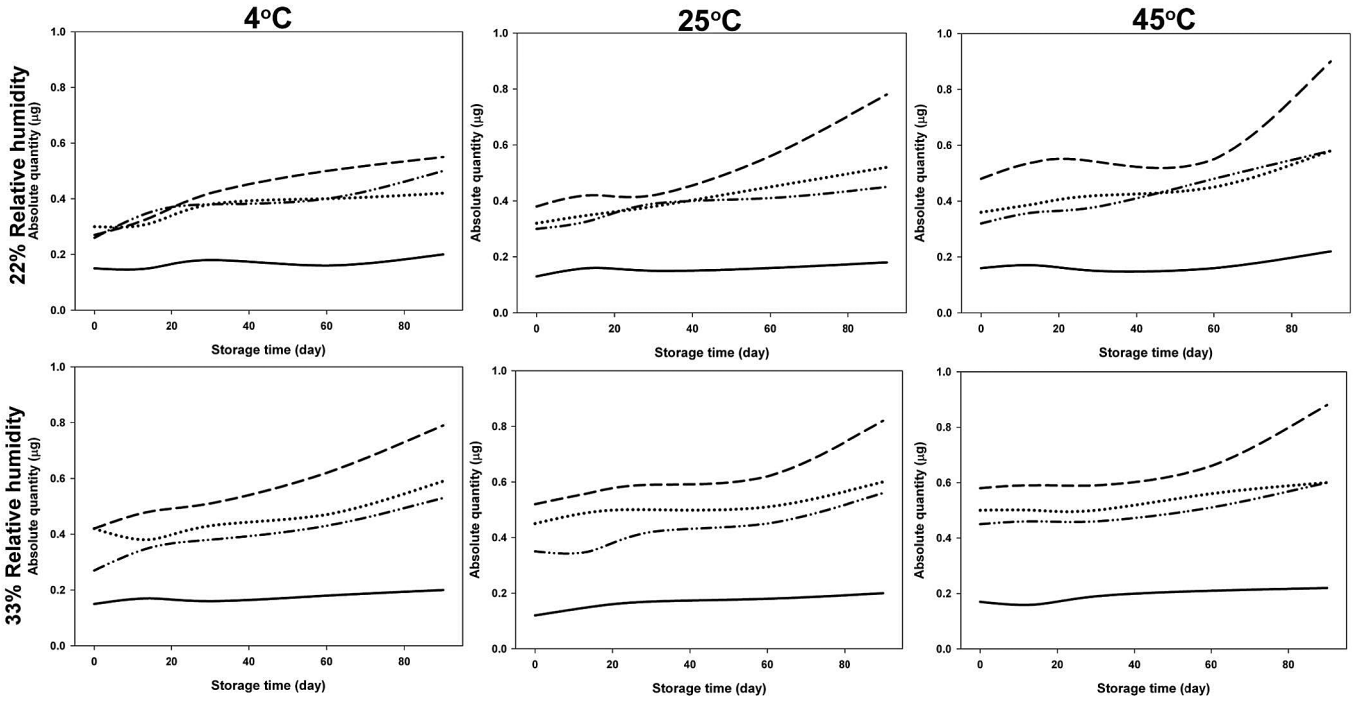

Fig. 7. Changes in absolute quantity (μg) of non-reducible aggregates during storage of native-WPC (solid line), sweet-WPC (dotted line), acid-WPC (dashed line) and salty-WPC (dashed-dotted line) at storage temperatures of 4, 25 and 45 °C and relative humidity of 22 and 33%, as obtained from image analysis of reducing SDS-PAGE.

Table 2
Reaction rate, reaction order, rate constant (K_n) activation energy (E_o) and pre- and pre-exponential factor (K_0) of β-LG disappearance in different WPCs during storage.

WPC	RH (%)	Temperature (°C)	Reaction rate for β-LG (μg/day)	Order of reaction	K_n ((g Kg^{-1})$^{(1-n)}$ day^{-1})	E_o (kJ mol^{-1})	Ink_0 ((g Kg^{-1})$^{(1-n)}$ day^{-1})
Native	22	4	0.09^{e}	1.4^{a}	7.0×10^{-5} ^{a}		
		25	0.18^{f}	1.0^{b}	1.2×10^{-3} ^{b}	96.3^{a}	32.2^{a}
		45	0.29^{g}	0.7^{c}	1.5×10^{-2} ^{c}		
	33	4	0.11^{h}	1.3^{d}	1.4×10^{-4} ^{d}		
		25	0.15^{i}	1.2^{e}	4.7×10^{-4} ^{e}	97.6^{b}	32.9^{b}
		45	0.39^{j}	0.6^{f}	3.9×10^{-2} ^{f}		
Sweet	22	4	0.13^{a}	1.2^{g}	2.8×10^{-4} ^{g}		
		25	0.22^{n}	0.9^{h}	3.2×10^{-3} ^{h}	58.3^{c}	17.3^{c}
		45	0.24°	0.8^{i}	6.9×10^{-3} ^{i}		
	33	4	0.16^{p}	1.1^{j}	5.4×10^{-4} ^{j}		
		25	0.19^{l}	0.9^{k}	2.6×10^{-3} ^{k}	41.2^{d}	10.5^{d}
		45	0.24^{q}	0.8^{l}	5.3×10^{-3} ^{l}		
Salty	22	4	0.14^{b}	1.2^{m}	5.8×10^{-4} ^{m}		
		25	0.16^{k}	1.1^{n}	6.8×10^{-4} ^{n}	23.1^{e}	2.4^{e}
		45	0.20^{l}	1.0°	$2.2 \times 10^{-3}$$^{\circ}$		
	33	4	0.07^{d}	1.7^{p}	3.3×10^{-5} ^{p}		
		25	0.08^{m}	1.6^{q}	5.9×10^{-5} ^{q}	31.9^{f}	3.4^{f}
		45	0.11^{h}	1.4^{r}	2.1×10^{-4} ^{r}		
Acid	22	4	0.12^{a}	1.4^{s}	1.3×10^{-4} ^{s}		
		25	0.13^{a}	1.4^{t}	1.9×10^{-4} ^{p}	13.2^{g}	3.2^{g}
		45	0.14^{b}	1.3^{u}	2.8×10^{-4} ^{t}		
	33	4	0.06^{c}	1.9^{v}	1.7×10^{-6} ^{u}		
		25	0.06^{d}	1.9^{w}	4.5×10^{-6} ^{v}	54.5^{h}	10.2^{h}
		45	0.10^{e}	1.6^{x}	3.8×10^{-5} ^{w}		
SEM			0.001	0.0	1.4×10^{-3}	0.1	0.04

[a-x] Different lowercase superscripts in the same column depicts the significant differences between means of kinetic parameters. Results are expressed as means of two independent replications. SEM = Standard error of the mean.

In acid-WPC, disulphide linked aggregates gradually increased throughout the storage with subsequent disappearance of β-LG A, β-LG B and α-LA (Figs. 5 and 6). Rapid involvement of β-LG A was observed in aggregation, compared to β-LG B (Oldfield et al., 1998), mainly due to disulphide bridging via Val_{118} in β-LG A as compared to Ala_{118} in β-LG B, located in β-strand H, creating a less favorably packed core (McSweeney and Fox, 2013). This in turn increases the flexibility of β-LG A and exposes the free thiol group. Compared to other WPCs, involvement of α-LA in protein aggregation was greater in acid-WPC. Less amount of water might have restricted the availability of Ca^{2+} to the binding site of α-LA, preventing protein stabilization and thus resulting in prevalence of less stable apo-α-LA. The contribution of caseins in disulphide bonding was apparent only at 45 °C and 33% RH conditions, although a significantly higher amount of caseins was present in acid-WPC than in other WPCs (Nishanthi et al., 2017a). A diffuse region was observed in the region between caseins and BSA (Mw~50 kDa) in the samples stored at 45 °C and two RHs under reducing PAGE conditions (Fig. 6), suggesting a possible aggregation between caseins and β-LG and/or α-LA via non-reducible covalent bonds. In addition to reducible aggregates, non-reducible aggregates likely created by lactosylation with Mw > 250 kDa were observed in acid-WPC under all storage conditions (Fig. 6). The extent of aggregation was clearly temperature and RH dependent (Fig. 7) and predominantly involved α-LA, as evidenced by the reaction rates (Table 3). Out of twelve lysine residues in α-LA, maximum of 6 residues (Lys_{24}, Lys_{112}, Lys_{117}, Lys_{127}, Lys_{133} and Lys_{141}) are lactosylated easily (Le et al., 2012).

Similar to other WPCs, salty-WPC contained disulphide linked protein aggregates with Mw > 250 kDa (Figs. 5 and 6). The aggregation increased with the storage time, achieving the maximum at 45 °C and 33% RH. Both β-LG A and β-LG B participated in disulphide bonding, however, β-LG A completely disappeared after 14 days, suggesting a high reaction rate (Oldfield et al., 1998). Involvement of α-LA in disulphide bonding was observed with extent clearly dependent of the storage temperature and time with no clear association with humidity. Thus, thiol/disulphide interchange reactions between β-LG and α-LA could be the main possible aggregation pathway. In absence of a reactive thiol group, thiol/disulphide interchange reactions occur through the disulphide bonds of Cys_6-Cys_{120} and Cys_{28}-Cys_{111} located on the surface of α-LA, which form new disulphide bonds with Cys_{66} and Cys_{160} located on the surface of β-LG. Compared to other WPCs, salty-WPC contained a high concentration of lactoferrin (LF) (Mw~87 kDa),

Table 3
Reaction rate, reaction order, rate constant (Kn) activation energy (E_0) and pre-exponential factor (K_0) of α-LA disappearance in acid-WPCs during storage.

WPC	RH (%)	Temperature (°C)	Reaction rate for α-LA (μg/day)	Order of reaction	K_n ((g Kg^{-1})$^{(1-n)}$ day^{-1})	E_0 (kJ mol^{-1})	Ink_0 ((g Kg^{-1})$^{(1-n)}$ day^{-1})
Acid	22	4	0.15^{a}	1.6^{a}	9.4×10^{-5} ^{a}		
		25	0.19^{b}	1.5^{b}	1.9×10^{-4} ^{b}	25.3 ± 0	1.7 ± 0
		45	0.20^{c}	1.5^{c}	3.9×10^{-4} ^{c}		
	33	4	0.13^{d}	1.7^{d}	6.4×10^{-5} ^{d}		
		25	0.17^{e}	1.7^{e}	8.7×10^{-5} ^{e}	17.4 ± 0.1	2.2 ± 0.04
		45	0.18^{f}	1.6^{f}	1.7×10^{-4} ^{f}		
SEM			0.001	0.0	7.9×10^{-4}		

[a-f] Different lowercase superscripts in the same column depicts the significant differences between means of kinetic parameters. Results are expressed as means of two independent replications. SEM = Standard error of the mean.

which did not participate in covalent aggregation under all storage conditions. On the other hand, BSA was greatly involved concomitantly with the rise in storage temperature, humidity and time. Due to a free thiol group at Cys_{34} and seventeen disulphide bonds in its primary structure, BSA participates in both disulphide bridging and thiol/disulphide interchange reactions with β-LG (Matsudomi et al., 1994) and α-LA (Matsudomi et al., 1993). Similar to sweet-WPC, salty-WPC contained aggregates linked via covalent non-disulphide bonds in the region of BSA-LF with approximate Mw of 71–72 kDa. Furthermore, the amount of large aggregates (Mw ≥ 250 kDa) formed via lactosylation increased in parallel with the storage temperature, humidity and time.

In four WPCs, type of proteins and interactions involved in protein aggregation vary with the type of powder, storage conditions and time. The main protein involved in disulphide and non-disulphide covalent aggregation was β-LG. Therefore, kinetic parameters of β-LG were estimated to thoroughly understand the differences in behavior of β-LG in these WPCs.

3.3. Kinetics of β-LG disappearance

Kinetic parameters of β-LG disappearance in different WPCs during storage are presented in Table 2. Disappearance of β-LG follows the common reaction kinetic principles. A substantial reaction occurs if an adequate number of β-LG molecules with sufficient activation energy (E_o) collide at an adequate frequency while maintaining specified molecular orientation to form disulphide or other covalent bonds. Rate of disappearance of β-LG ($R_{\beta\text{-LG}}$) in native-WPC and sweet-WPC was directly related to the storage temperature and humidity, being the greatest at 45 °C and 33% RH. Elevated temperature increases the number of reactive β-LG molecules with exposed free thiol group of Cys_{121}. High RH increases water activity thus favors thermal energy transition and molecular motions, facilitating formation of reactive monomers. In contrast, $R_{\beta\text{-LG}}$ of salty-WPC and acid-WPC decreases as RH increases. This observation suggests a greater interference of compositional constituents present in these two WPCs on $R_{\beta\text{-LG}}$. As a result of the low pH in presence of high concentration of LA in acid-WPC (Nishanthi et al., 2017a), dimerization of β-LG and formation of octamers (Townend and Timasheff, 1960) occurs as evidenced by the FTIR spectra and SDS^{NR} PAGE patterns. Due to the large structure of octamer, number of reactive sites exposed to the surface is low, leading to low $R_{\beta\text{-LG}}$. Further, the osmotic environment with the presence of LA and Ca^{2+} retain water molecules, preventing protein hydration. As a result, in low RH, protein-protein interactions dominate, limiting the mobility of WPs. Ca^{2+} on the other hand, forms salt bridges between carboxyl groups of amino acids (Zhu and Damodaran, 1994), restricting the collision in required orientation for permanent covalent bonding. Furthermore, anionic lactate actively increases the negative charge at the dimer interface, stabilizing the β-LG dimers (Mercadante et al., 2012) and thereby, prevent the formation of reactive monomers. Salty-WPC, is characteristically high in Na^+ (approximately 0.5 M), which exerts an osmotic pressure of ~0.005 bars, nearly 3 times greater compared to that of sweet-WPC. Under these conditions, Na^+ ions are preferentially excluded from the protein surface which affects the binding of water to the protein (Arakawa and Timasheff, 1982). In addition, Na^+ itself can interact with proteins through salt bridges and charge shielding, subsequently inducing conformational changes and restricting the mobility of the proteins. Influence of compositional variations of acid- and salty-WPCs on β-LG reaction kinetics is further evident by the reaction order and E_o. Reaction orders of acid- and salty-WPCs were approximately closer to 2 under all storage conditions, whereas, that in native- and sweet-WPCs was approximately closer to 1. Furthermore, native-WPC required the greatest E_o compared to other WPCs, implying that β-LG in native-WPC are still in their native form, due to absence of severe thermal treatments during its manufacturing process. Moreover, native-WPC consisted of 84% proteins on dry basis, implying the lack of influence of other whey components on the β-LG structure. Compared to native- and sweet-WPC, E_o was low in acid- and salty-WPCs. Compositional variations and heat treatments applied in manufacturing processes of acid- and salty-WPCs partially denature the β-LG, lowering the E_o.

In addition to β-LG, α-LA was also involved in protein aggregation in acid-WPC (Table 3), with a higher rate of disappearance ($R_{\alpha\text{-LA}}$) than β-LG. This observation contradicts the assumption that in Ca^{2+} rich environment of acid-WPC α-LA should demonstrate the least reactivity due to the high structural stability attained with the Ca^{2+} bound holo-form. However, low E_o established for α-LA in the current study suggests structural instabilities. Perhaps folding intermediates of α-LA with a low affinity for Ca^{2+} (McSweeney and Fox, 2013) are more common than the native form in acid-WPC. Rate of disappearance of α-LA ($R_{\alpha\text{-LA}}$) varied as 4 < 25 < 45 °C and 33 < 22%. At high temperature, unfolding rate of α-LA increased, shifting the equilibrium of α-N ⇌ α-U towards the right side (Oldfield et al., 1998), in which state α-LA becomes more prone to engage in thiol/disulphide interchange reactions with reactive β-LG.

4. Conclusion

Origin, composition and storage conditions clearly affected the properties of WPCs examined. Protein aggregation occurred predominantly through covalent crosslinking under all storage temperatures, while higher temperatures accelerated aggregation. Humidity had a variable effect on the protein aggregation, mainly based on the type of WPC. Disulphide and non-disulphide covalent crosslinking of β-LG was the main aggregation pathway in all WPCs, whereas, involvement of α-LA, BSA and caseins depended on the type of WPC. In parallel to the protein aggregation, disappearance of β-LG followed a first-order reaction in native-WPC and sweet-WPC, while it was closely a second-order reaction in acid-WPC and salty-WPC. Compositional variations associated with origin of acid-WPC and salty-WPC facilitates the β-LG loss during the storage. According to the result of the current study, 4 °C can be considered as the optimal storage temperature for all types of WPCs, while determination of optimal relative humidity requires further understanding about effects of storage on physical properties and functional characteristics of these powders.

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Chapter 6: Physical properties of selected spray dried whey protein concentrate powders during storage

Chapter 6 presents the physical changes of sweet, acid and salty-whey protein concentrates powders subjected to several storage conditions including three different storage temperatures (4, 25 and 45 °C) and two relative humidity levels (22 and 33%) over a storage period of 90 days.

The paper entitles “Physical properties of selected spray dried whey protein concentrate powders during storage” by Manjula Nishanthi, Jayani Chandrapala and Todor Vasiljevic has been published in the peer-reviewed journal “Journal of Food Engineering” (2018), 219: 111 – 120. https://doi.org/10.1016/j.jfoodeng.2017.09.021

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DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: Manjula Nishanthi Kottahachchi Kankanamge

Signature: [redacted]

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In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Manjula Kankanamge	80	Concept development, research question, hypothesis and manuscript preparation
Dr. Jayani Chandrapala	10	Concept development, research question, hypothesis and contribute in writing manuscript
Prof. Todor Vasiljevic	10	Concept development, statistical analysis, artwork preparation, contribute in writing manuscript and submission to journal

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The undersigned certify that:

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Physical properties of selected spray dried whey protein concentrate powders during storage

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ABSTRACT

Storage conditions may compromise stability of whey proteins in dry state, which is also influenced by their inherent composition. Thus, physical characteristics of native, sweet, acid and salty-WPC powders were analysed during storage at several temperatures (4, 25, 45 °C) and relative humidities (22, 33%) for a period of 90 days. Particle surface of native, sweet and acid-WPCs was dominated by proteins under all storage conditions, while fat and minerals prevailed on the surface of salty-WPC. Compared to native- and sweet-WPCs, origin of acid- and salty-WPCs influenced these streams to be rich in minerals, primarily accumulated in the particle core. Hydrophilic nature of the core impacted redistribution of proteins within the particle during storage. Compared to native- and sweet-WPC, particles were cohesively arranged in acid- and salty-WPC, which in turn changed physical properties such as particle size and surface charge. Elevated storage temperatures induced protein denaturation, melting of surface free fat and lactose crystallization in WPCs, while humidity regulated the molecular mobility in these reactions.

1. Introduction

Growing demand for high protein foods has resulted in an exponential rise in the production of several dairy products including strained yoghurt, soft and various hard cheeses. While profits have increased, so has the cost of the production as most of these products undergo numerous steps in processing, some of which generate by-product streams, in general termed whey. For instance, manufacturing of strained yoghurt, cream and fresh cheeses releases acid whey as a by-product, while production of hard cheeses involves the release of sweet whey. A salting step in the cheese processing also results in release of so-called salty whey. Due to constant rise in demand for strained yoghurt, hard and fresh cheeses and thus increase in the processed and released volumes of whey, it has become an imperative for the industry to find ways to utilize these streams.

Attempts to utilize existing technologies to manufacture whey protein concentrates (WPC) out of these whey streams have been hindered by their inherent compositional differences in comparison to sweet whey, specifically high salinity in salty whey or high acidity in acid whey (Nishanthi et al., 2017c). Calcium-based lactate salts in acid whey and sodium-based salts in salty whey are some of the reasons for a rapid membrane fouling, extended filtration time and cost. In addition, lactic acid present in a greater concentration in acid whey hinders lactose crystallization and forms hygroscopic clumps during drying (Chandrapala et al., 2015).

Apart from several manufacturing difficulties, storage conditions of these WPC powders may affect functional properties and thus further applications. Proper storage conditions are thus of critical importance in order to maintain the original in-solution physio-chemical and functional properties of these proteins throughout the shelf life without quality deteriorations. Occurrence of these changes is highly dependent on storage conditions and composition of powders. Out of many possible chemical reactions, Maillard reaction draws a high attention due to colour changes and flavour deterioration. For instance, storage of a sweet whey protein powder at elevated temperatures resulted in non-enzymatic Maillard-type reactions reducing its nutritional and functional value, compared to those stored at room temperature (Dattatreya and Rankin, 2008; Sithole et al., 2005). Storage of native whey protein powder at 40 °C exhibited extensive binding of lactose to the whey proteins and thereby formation of Maillard reaction products. As a consequence, the net charge of whey proteins increased while the fraction of native protein decreased (Norwood

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et al., 2016a). Furthermore, the level of lactosylated proteins increased faster during the initial 15 days of storage at 40 °C but remained stable for the next 3 months afterwards. After this period, a level of lactosylated proteins declined in response to a concomitant increase in protein denaturation (Norwood et al., 2016a).

Denaturation of whey proteins is yet another type of reaction that takes place in response to changes during storage. Conformational changes affect a number of important physical characteristics of whey powders including particle size, surface charge, colour, thermal behaviour and morphological character. Native whey protein isolates stored at 20 °C and 23% relative humidity (RH) experienced ~7% denaturation of β-LG over 12 months period while extent of denaturation increased to ~33% when the temperature rose to 40 °C (Norwood et al., 2016b).

Apparently significant and complex inter- and intramolecular interactions take place among whey proteins in response to varying storage conditions leading to denaturation and aggregation (Nishanthi et al., 2017b). Extent of these interactions and rate of changes depend on a cumulative effect of storage conditions and compositional matrix of WPC powders (Nishanthi et al., 2017b) likely affecting their physical characteristics and ultimately functional performances. Due to their abundance and importance, sweet- and native-WPCs have been investigated extensively under various storage conditions. On the other hand, emerging whey streams and their respective WPCs such as acid and salty-WPC powders have not been in-depth assessed in order to establish possible physical changes that could potentially impact on their functionality and applicability. Therefore, the main aim of the current study was to establish physical changes of sweet-, acid- and salty-WPC powders subjected to several storage conditions including three different storage temperatures (4, 25 and 45 °C) and two RHs (22% and 33%) over a storage period of 90 days. Native-WPC was used as the reference.

2. Materials and methods

2.1. Sample preparation

Four WPCs (native-WPC, sweet-WPC, acid-WPC and salty-WPC) obtained from our previous study (Nishanthi et al., 2017a) were used in the storage trial. As reported in that study, average composition of four WPCs on dry matter basis was: native-WPC – 84.1% protein and 4% lactose; sweet-WPC – 61.8% protein and 17% lactose; acid-WPC – 67% protein, 24% lactose, 7% lactic acid (LA) and 5% Calcium (Ca); salty-WPC – 10.9% protein, 27% lactose and 12% sodium (Na). The average pH of native, sweet, acid and salty-liquid WPCs was 6.5, 6.4, 4.2 and 5.5, respectively. The WPC powders were fully dehydrated by a vacuum desiccation over phosphorous pentoxide at room temperature (25 °C) for 4 weeks.

2.2. Storage trial

Approximately 3 g of each WPC was placed in standard desiccators (with a porcelain disc size of 250 mm) with controlled relative humidity (RH) of 22 or 33%, sealed and placed in temperature controlled cabinets at three temperatures (4, 25 or 45 °C). The equilibrium RH in each desiccator was maintained using saturated salt slurries containing potassium acetate (22 ± 2%) or magnesium chloride (33 ± 1%). At 4, 25 and 45 °C, magnesium chloride maintained a RH of 34, 33 and 32%, while potassium acetate maintained RH of 24, 23 and 23%, respectively. Five desiccators were prepared for one temperature/humidity combination as sampling was conducted at 5 time points representing 0, 14, 30, 60 and 90 days. First sampling (0 day) was performed after allowing for a 2-week equilibration period from the initial date of the storage trial in order to ensure that the required RH was achieved. After opening desiccators at the required time point, the analyses were carried out within 3 days.

2.3. Analysis

2.3.1. Particle size distribution

Particle size distribution of WPCs were measured as described previously (Nishanthi et al., 2017a) using a Malvern Master Sizer S (Malvern Instruments, Malvern, UK). Isopropanol was used as the dispersant, considering 1.45 and 1.37 as the refractive index (RI) of whey protein concentrate and isopropanol, respectively. The average of three measurements was taken and the size is expressed as D (0.5), the volume –weighted median diameter.

2.3.2. Surface charge

Surface charge of WPCs were determined by laser-doppler electrophoresis using the Malvern Zetasizer Nano ZSP while ethanol (100% w/v) with RI of 1.36 was used as the dispersant (Nishanthi et al., 2017a).

2.3.3. Differential scanning calorimetry (DSC)

A DSC 1 STARe System (Mettler Toledo, Schwerzenbach, Switzerland) purged with nitrogen (50 ml/min) was used to determine the denaturation temperatures and enthalpy of powders. About 5–10 mg of WPC was scanned in hermetically sealed 40 μL DSC aluminium pans (Mettler Toledo, Schwerzenbach, Switzerland). An empty aluminium pan was used as a reference. All samples were scanned with a starting temperature of 25 °C and end temperature of 250 °C at a heating rate of 10 °C min^{-1}.

2.3.4. X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) was carried out to determine the elemental composition of selected powder samples using a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) with a monochromated aluminium X-ray source (10 mA, 15 kV) within 10 nm of the surface as described by Nishanthi et al. (2017a). The elemental ratios were further interpreted as the amounts of fat, protein and lactose according to the method described by Faldt et al. (1993). Not all samples at temperature/humidity combinations were analysed by XPS. Selection of samples was carried out based on the variation of their surface charge.

2.3.5. Scanning electron microscopy (SEM)

Visualization of microstructure of selected samples of WPCs was performed using the JEOL NeoScope JCM-5000 A scanning electron microscope (SEM) (Tokyo, Japan) as previously described by Nishanthi et al. (2017a). Similar to XPS analysis, only selected samples were analysed with selection carried out based on the variation of surface charge.

2.4. Statistical analysis

The experiments were organized in a randomized split plot blocked design with 3 main factors: a type of WPC powder as the main plot, and temperature and RH as subplots. The replications served as blocks. Replicated samples were sub sampled and statistically analysed using the General Linear Model (GLM). The level of significance was pre-set at $p < 0.05$.

3. Results

3.1. Particle size

Table 1 depicts changes of an average particle size of WPCs over the storage period of 90 days under different storage conditions. Particle size of WPC was significantly ($p < 0.05$) influenced by all experimental factors including storage temperature, humidity, time and type of WPC. At 4 and 25 °C, particle size of native-WPC gradually increased from day 0, reaching its maximum at the 60th day and subsequently decreasing afterwards. In contrast, at 45 °C, particle size of native-WPC gradually increased throughout the storage period of 90 days. Sweet-WPC showed a similar pattern of particle size increment to that of native-WPC. From the beginning of storage, particle size of sweet-WPC gradually increased and reached its maximum by the 30th day of storage at 4 °C and by the 60th day at 25 and 45 °C. Under all storage conditions, particle size declined after 60th day, however comparatively larger particles were formed at 45 °C. Different to both native- and sweet-WPC, particle size of acid-WPC gradually decreased during the storage at 4 °C. In contrast, at 25 and 45 °C, the particle size of acid-WPC gradually increased during the storage, resulting in large particles under 33% RH compared to those under 22% RH. Compared to other WPCs, particles of salty-WPC were large. Under all storage conditions, particle size decreased from the beginning to mid-storage and increased afterwards. The particle size increment after mid-storage was prominent in samples stored under 33% RH, compared to those under 22% RH.

3.2. Surface charge

Table 2 shows variations of surface charge of WPCs over the storage period of 90 days under different storage conditions. Surface charge of WPCs was significantly ($p < 0.05$) impacted by all experimental factors. Surface charge of native-WPC powder varied following a similar trend to that of the particle size: being negative at the start of storage, becoming progressively positive reaching a maximum at 60th day and subsequently decreasing. Decline in surface potential after 60th day was prominent at 45 °C and 33% RH, with a negative charge of −5.8 mV. Sweet-WPC also showed a comparatively large negative charge at the beginning of the storage, which became positive at the 60th day, and then reverted to a negative charge by the end of storage. In contrast to native- and sweet-WPC, surface charge of acid-WPC at the start of the storage was positive under all storage conditions. This charge became weakly negative after 14 days, followed with another change between 30 and 60 days. At the end of storage, the particles were again negatively charged. As compared to other WPCs, surface of salty-WPC particles remained positively charged under all storage conditions throughout the storage.

3.3. Denaturation enthalpy and peak temperature

Storage temperature, RH, time and type of WPC significantly ($p < 0.05$) influenced the variation of the enthalpy and peak temperature of WPCs denaturation (Tables 3 and 4). At the beginning of the storage, native-WPC exhibited a significantly higher

Table 1
Particle size distribution of WPC powders, dispersed in isopropanol and analysed by a Malvern Mastersizer, stored under storage temperature of 4, 25 and 45 °C and relative humidity of 22 and 33% for a storage period of 90-day.

Temperature (°C)	Humidity (%)	Time (days)	Particle size (μm)			
			Native-WPC	Sweet-WPC	Acid-WPC	Salty-WPC
4	22	0	61.8	49.3	73.2	230.0
		14	62.0	49.2	73.2	178.3
		30	67.1	55.4	67.4	180.4
		60	80.2	48.2	62.8	130.5
		90	65.9	47.2	62.2	152.0
	33	0	61.6	42.2	76.3	227.4
		14	63.1	47.8	66.4	191.5
		30	68.1	55.5	61.5	152.6
		60	70.5	43.9	61.2	140.8
		90	56.3	47.6	58.6	219.3
25	22	0	60.8	47.4	53.7	173.4
		14	63.2	52.8	59.1	168.4
		30	67.1	39.1	60.7	136.0
		60	68.9	50.0	60.4	132.3
		90	62.3	48.1	67.6	145.4
	33	0	65.9	42.9	48.0	190.7
		14	67.9	42.9	51.1	183.8
		30	70.1	49.2	64.4	168.1
		60	73.9	49.5	64.1	165.1
		90	62.2	45.2	70.7	217.7
45	22	0	64.4	49.3	62.4	165.0
		14	64.6	43.4	63.9	164.0
		30	66.7	49.1	64.4	127.9
		60	69.5	54.5	67.8	142.0
		90	75.6	50.4	68.3	145.5
	33	0	63.0	44.7	64.4	189.9
		14	64.4	48.4	68.4	159.8
		30	65.9	51.3	68.9	128.7
		60	68.9	53.3	70.2	194.2
		90	70.6	50.8	91.6	223.3
SEM			16.5			

Values are means of at least 4 independent observations (sample size $n \geq 4$); SEM: Pooled standard error of the mean, WPC: Whey protein concentrate.

Table 2
Surface charge of WPC powders, dispersed in ethanol and analysed by Malvern Zetasizer, stored under storage temperature of 4, 25 and 45 °C and relative humidity of 22 and 33% for a storage period of 90-days.

Temperature (°C)	Humidity (%)	Time (days)	Surface charge (mV)			
			Native-WPC	Sweet -WPC	Acid-WPC	Salty-WPC
4	22	0	−9.3	−23.4	9.0	9.8
		14	−6.2	−9.3	−3.6	6.6
		30	11.0	−3.4	4.3	7.8
		60	12.5	11.6	12.0	7.8
		90	8.7	−11.1	−5.2	7.7
	33	0	−6.3	−6.1	2.9	10.1
		14	−2.8	−8.1	−1.8	7.3
		30	4.1	−4.5	4.1	11.5
		60	4.4	10.9	17.4	9.9
		90	7.8	−13.3	−3.9	7.3
25	22	0	−3.8	−14.8	11.9	6.8
		14	5.5	−7.4	−5.3	13.1
		30	14.7	−5.2	7.0	6.8
		60	25.2	17.7	8.9	7.8
		90	5.2	−8.2	−3.7	11.7
	33	0	−5.4	−9.9	6.8	7.1
		14	8.6	−13.1	−3.6	8.3
		30	12.1	6.2	5.8	8.2
		60	16.1	14.9	23.8	9.4
		90	8.1	−14.5	−5.1	10.4
45	22	0	−5.5	−16.2	16.8	9.4
		14	−4.7	−6.5	9.3	14.3
		30	8.6	8.3	17.6	10.6
		60	32.4	16.8	18.0	11.6
		90	3.3	−12.9	−7.1	10.3
	33	0	−7.4	−12.2	4.6	11.3
		14	−4.1	−9.3	−4.6	11.7
		30	7.0	3.2	3.7	7.8
		60	14.5	9.4	9.3	13.3
		90	−5.9	−8.7	−5.8	9.1
SEM			0.7			

Values are means of at least 4 independent observations (sample size $n \geq 4$); SEM: Pooled standard error of the mean, WPC: Whey protein concentrate.

Table 3
Denaturation enthalpy of WPC powders, analysed by differential scanning calorimetry (DSC), stored under storage temperature of 4, 25 and 45 °C and relative humidity of 22 and 33% for a storage period of 90-days.

Temperature (°C)	Humidity (%)	Time (days)	Denaturation enthalpy (J/g)			
			Native-WPC	Sweet-WPC	Acid-WPC	Salty-WPC
4	22	0	−93.0	−155.5	−183.8	−87.3
		30	−74.5	−121.5	−113.3	−50.5
		90	−62.6	−118.1	−156.1	−30.8
	33	0	−141.4	−195.9	−158.6	−96.9
		30	−102.8	−116.7	−172.7	−96.3
		90	−101.7	−85.9	−180.6	−84.6
25	22	0	−87.8	−177.4	−88.6	−74.8
		30	−83.0	−148.5	−41.0	−66.3
		90	−71.2	−103.9	−33.6	−43.7
	33	0	−158.0	−159.6	−63.2	−90.9
		30	−77.5	−104.8	−57.6	−57.8
		90	−59.9	−49.3	−48.8	−41.5
45	22	0	−75.5	−133.6	−61.2	−75.8
		30	−67.0	−57.4	−43.4	−73.1
		90	−54.3	−11.1	−24.5	−54.8
	33	0	−101.2	−163.4	−29.5	−60.7
		30	−65.2	−136.5	−22.8	−47.4
		90	−43.8	−76.2	−18.9	−46.6
SEM			12.9			

Values are means of at least 4 independent observations (sample size $n \geq 4$); SEM: Pooled standard error of the mean, WPC: Whey protein concentrate.

Table 4
Denaturation (peak) temperature of WPC powders stored under storage temperature of 4, 25 and 45 °C and relative humidity of 22 and 33% for a storage period of 90-days.

Temperature (°C)	Humidity (%)	Time (days)	Denaturation temperature (°C)			
			Native-WPC	Sweet-WPC	Acid-WPC	Salty-WPC
4	22	0	114.3	157.8	159.3	137.2
		30	157.9	163.9	153.1	162.3
		90	125.5	164.0	153.7	175.2
	33	0	155.3	156.5	145.7	161.7
		30	156.0	143.5	140.6	161.2
		90	135.9	120.0	153.2	162.2
25	22	0	157.2	151.7	154.8	163.8
		30	167.1	155.7	136.2	162.9
		90	147.2	157.4	132.5	156.7
	33	0	126.6	151.3	142.6	170.6
		30	152.8	146.1	148.4	160.5
		90	133.7	110.8	123.0	152.5
45	22	0	125.5	157.7	155.9	159.3
		30	148.9	157.0	155.3	170.6
		90	164.3	158.3	130.1	171.7
	33	0	115.9	154.3	161.5	156.1
		30	157.8	154.7	158.5	168.5
		90	164.1	137.0	132.9	173.5
SEM			0.80			

Values are means of at least 4 independent observations (sample size $n \geq 4$); SEM: Pooled standard error of the mean, WPC: Whey protein concentrate.

denaturation enthalpy under 33% RH compared to that under 22%. As storage progressed, denaturation enthalpy decreased gradually under all storage conditions, being the lowest at 45 °C for both RHs at the end of storage. Peak temperature of endothermic denaturation continuously increased at 45 °C for both RHs, while at 4 and 25 °C, it maximized at day 30. Denaturation enthalpy of sweet-WPC was highest at the beginning of the storage, and gradually decreased as storage progressed, remaining high for the samples stored at 4 °C, as compared to those at 25 or 45 °C. Endothermic peak temperature of sweet-WPC showed a clear difference based on the RH, increasing gradually under RH of 22%, gradually decreasing under RH of 33% over the storage period. In acid-WPC, denaturation enthalpy was significantly ($p < 0.05$) higher at 4 °C, compared to that at 25 and 45 °C and gradually declined at higher temperatures. Similar trend was observed for the peak temperature for this sample. Compared to native and sweet-WPCs, denaturation enthalpy was significantly lower in salty-WPC for all storage conditions and gradually declined during storage. Peak temperature of denaturation gradually decreased at 25 °C, while it increased at 4 and 45 °C with the storage time.

3.4. Surface composition and hydrophobicity

Table 5 shows the variation of surface composition of WPCs in terms of the major components including protein, lactose, fat and minerals. It further established hydrophobicity of the particle surface, estimated by the elemental bonding state of carbon (C) in the near surface region (10 nm) of the particle. It was presumed that the increased non-polar bonds (C-C) at the surface are associated with the increased hydrophobicity of the particle surface (Fyfe et al., 2011). It is important to note that only selected results of the surface composition are mentioned here, as the sample selection for XPS analysis was based on variations in the surface charge of powders. In native-WPC powder, particle surface was dominated by proteins under all storage conditions. Storage at 4 °C exhibited a comparatively high increment (~3%) of surface protein over 90 days, as compared to that at 25 and 45 °C. At 45 °C and 33% RH significantly high fat coverage was determined on the particle surface, which gradually declined over 90 days. Native-WPC had a minimal lactose coverage on its particle surface, which was lactose-free at 45 °C and 33% RH. Surface of native-WPC mainly consisted of Na, which content slightly increased over the storage period, under all storage conditions. As the storage progresses, the hydrophobicity of native-WPC gradually increased at 4 and 25 °C, however progressively decreased at 45 °C with the storage time.

Although the particle surface of sweet-WPC powder was dominated by proteins, their concentration was substantially lower compared to that of the native-WPC, likely due to comparatively high lactose and fat content in sweet-WPC. During the storage for 90 days, surface protein coverage on sweet-WPC declined by 3.8%, while fat coverage increased by 3.3%, at 4 °C. During the same period, protein coverage on sweet-WPC increased by 3.5% and 3.7%, while lactose coverage also increased by 3.3% and 1.7%, at 25 and 45 °C, respectively. In parallel, fat percentage on particle surface decreased by 6.8% and 5.4%, at 25 and 45 °C, respectively. Interestingly at 25 °C, hydrophobicity also gradually declined over time; however, at 45 °C, hydrophobicity increased substantially, although fat content on the particle surface concomitantly decreased.

Particle surface of acid-WPC was predominantly covered by proteins. Over the storage period, the protein coverage gradually increased by 5% and 3.5% at 4 and 45 °C, respectively, by replacing equal amounts of fat and lactose. In contrast, at 25 °C, protein content on the surface significantly decreased by 12.5%, providing space for extra 11.6% of fat deposition. Correlation between the surface fat coverage and surface hydrophobicity was observed only at 4 °C, where declining the fat coverage resulted in decreased hydrophobicity on the particle surface.

Compared to other WPCs, surface area covered by proteins was significantly lower in salty-WPC. This surface coverage was significantly reduced by 8% during storage only at 25 °C and 33% RH. Although fat was the main component on salty-WPC particle, no observable increase in fat coverage was noted under any storage condition. Lactose coverage increased slightly by 1.9%, at 25 °C and 33% RH. The second most abundant component on the surface was minerals, primarily Na, which content increased by 8% under 25 °C and 33% RH, while it remained fairly constant under other conditions. At the same time, hydrophobicity of the particle surface was low and remained constant at 25 °C and 33% RH. At 4 °C, salty-WPC

Table 5
Surface composition of WPC powders, determined by X-ray Photoelectron Spectroscopy (XPS), stored under storage 500 temperature of 4, 25 and 45 °C and relative humidity of 22 and 33% for a storage period of 90-days.

Temperature (°C)	Humidity (%)	WPC	Time (days)	Protein (%)	Lactose (%)	Fat (%)	Minerals (%)	Percentage of C-C bond
4	22	Native	0	94.5	1.64	3.1	0.8	46.4
			90	97.5	1.46	0	1.0	48.1
	22	Sweet	0	67	10.4	22.4	0.2	54.4
			60	63.6	12.0	24.2	0.2	55.7
			90	63.2	10.9	25.7	0.2	55.6
	22	Acid	0	71.7	9.8	17.8	0.7	53.9
			90	76.6	7.9	14.8	0.7	51.8
	22	Salty	0	13.1	22.2	40.0	24.8	49.6
			90	12.2	19.4	42.6	25.8	43.9
25	22	Native	0	92.4	1.6	4.7	1.1	45.6
			90	93.6	1.6	3.7	1.2	49.1
	33	Sweet	0	62.7	9.5	27.6	0.2	57.2
			30	62.7	12.1	25.0	0.2	55.2
			90	66.2	12.8	20.8	0.2	54.4
	33	Acid	0	71.1	4.3	24.4	0.2	51.3
			30	62.9	5.7	31.0	0.4	43.8
			90	58.6	5.2	36.0	0.2	38.9
	33	Salty	0	19.4	9.7	54.6	16.3	14.5
			90	11.4	11.6	52.7	24.3	14.6
45	33	Native	0	85.7	0	14	0.6	43.6
			30	86	0	13.4	0.6	41.3
			90	87.5	0.84	10.6	1.0	38.24
	22	Sweet	0	63.9	8.6	27.3	0.2	38.5
			30	63.1	9.9	26.8	0.2	47.6
			90	67.6	10.3	21.9	0.2	47.5
	22	Acid	0	71.9	6.8	20.8	0.5	45.7
			90	75.4	5.4	18.5	0.7	45.4
	22	Salty	0	17.5	20.9	39.5	22.1	28.3
			90	16.8	20.1	37.7	25.4	21.2

showed the highest hydrophobicity, where it was almost twice as that at 45 °C.

3.5. Particle morphology

Figs. 1 and 2 show the SEM images of different WPC stored under different storage conditions at the beginning and end of storage period, respectively. As mentioned above, only selected samples, based on their variation in the surface potential, were analysed for their characteristics. Particles of native-WPC powder were globular and free flowing at the start of storage. However, as storage progressed, particles agglomerated gradually, showing an intense aggregation at higher storage temperatures. Also, a substantial number of particles appeared ruptured at high storage temperatures. Compared to other WPCs, large and spherical particles were observed in sweet-WPC. At the start of storage, particles were a mixture of dented- and smooth-surface. During 90 days, particles ruptured, aggregated and punctured. In acid-WPC, particles had near-globular shape and dented-surfaces at the start of the storage. Over the storage, particles aggregated, mainly influenced by temperature. Similar to acid-WPC, particles of salty-WPC aggregated intensively at high storage temperatures, forming into clusters-like aggregates.

4. Discussion

Acid and salty whey are compositionally different to sweet whey mainly due to high content of lactic acid (LA) and minerals. These differences enforce different inherent protein characteristics in acid and salty liquid whey (Nishanthi et al., 2017c) and their liquid concentrates (Nishanthi et al., 2017a). Upon spray drying, these differences are further emphasised and depicted in surface composition of acid- and salty-WPC powders, rendering them substantially distinctive to those of sweet- or native-WPC powders (Nishanthi et al., 2017a). Surface of salty-WPC was characterized with the least protein coverage and highest content of fat, lactose and minerals compared to other WPCs. Acid-WPC contained intermediate levels of protein, lactose, fat and mineral coverage compared to native- and sweet-WPCs. During storage under elevated temperature and humidity, these compounds are activated and engaged in various chemical reactions and physical interactions resulting in re-distribution between the particle core and the surface.

Primary chemical change of proteins is their denaturation and subsequent aggregation. During storage at elevated temperature and humidity whey proteins intensively unfold exposing reactive sites allowing for interactions with nearby molecules (Nishanthi et al., 2017b). As the main whey protein, β-LG predominates this process engaging into interactions with α-lactalbumin (α-LA), caseins and bovine serum albumin. β-LG has the lowest thermal stability with a free thiol group and interiorly placed hydrophobic patches that favour aggregation. Linked by disulphide bridges and hydrophobic interactions, oligomers are formed, which then leads to formation of larger aggregates (Schmitt et al., 2010) depicted by rise in particle size of native- and sweet-WPC up to 60 days. Clustering of oligomers might reach a critical charge density after 60 days, thus limit further aggregation, instead a large number of small aggregates is formed (Norwood et al., 2017). As a result of protein

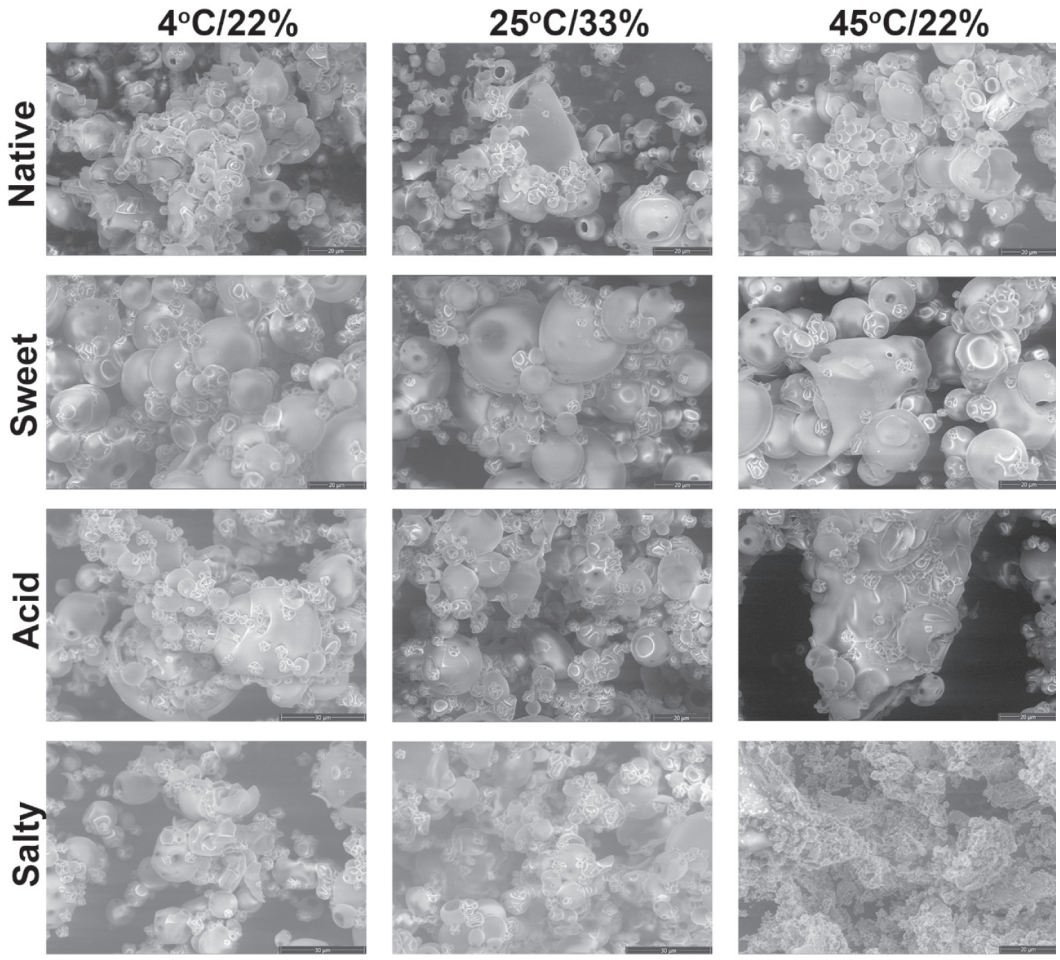

Fig. 1. Micrographs, obtained by scanning electron microscopy, of native, sweet, acid and salty-WPC powders equilibrated to 22 or 33% relative humidity at 4, 25 or 45 °C at the beginning of a 90-day storage period (0 day). The magnification was 3600x, with a bar indicating 20 μm length. The labelling of the columns denotes storage conditions including temperature (°C) and relative humidity (%), while the labelling of the rows depicts a type of the WPC powder.

denaturation, proteins lose their native state, which lowers their surface activity and likely influences their presence on a particle surface (Kim et al., 2009). In contrast, in absence of protein denaturation, proteins diffuse from the core to the surface due to their inherent surface activity. However, preferential diffusivity of milk components towards the particle surface is also governed by their particle size (Kim et al., 2003), with diffusivity descending in order of fat, caseins, whey proteins, lactose and minerals.

In addition to protein denaturation, Maillard polymerization is another chemical reaction that has been identified to change the surface composition during storage. The covalent bonding between carbonyl group of lactose and amino group of amino acids yield poorly characterised Maillard products leading to non-enzymatic browning. Six lysine residues (Lys_{24}, Lys_{112}, Lys_{117}, Lys_{127}, Lys_{133} and Lys_{141}) in α-LA (Le et al., 2012) and three lysine residues (Lys_{8}, Lys_{141} and Lys_{138}) in β-LG are lactosylated easily (Nishanthi et al., 2017b), therefore, likely the primary sites of the reaction. Maillard polymerization in turn affects the heat-induced protein aggregation, where formation of more Maillard products subsequently forms smaller aggregates (Norwood et al., 2017). The PAGE analysis reported in our previous study suggested that Maillard reaction based protein polymerization occurred in sweet-WPC towards the end of storage (Nishanthi et al., 2017b). Sweet-WPC contained 17% (w/w) of lactose, which appeared adequate to lactosylate a significant number of lysine residues in whey proteins, in turn resulting in an increased net negative charge (Wang and Ismail, 2012). Compared to native-WPC, greater surface area of sweet-WPC is covered by lactose at 25 and 45 °C, which increased concomitantly with the increasing protein coverage, suggesting a Maillard reaction. However, this could not be further confirmed by colour changes of powders since the colour measurement was not used in the analysis. Furthermore, lactose crystallization can also take place during storage, which possibly lead to variations in surface composition. Lactose in spray-dried powders may exist in the amorphous glassy state or in the crystalline form depending on the drying and storage conditions (Aguilar and Ziegler, 1993). Although the amorphous lactose acts as a dispersion medium for proteins and fat inside the particle, crystalline lactose separates these components, releasing fat to the particle surface (Moreau and Rosenberg, 1993). Therefore, under appropriate storage conditions that induce lactose crystallization, migration of fat from the core to the surface could be clearly observed.

Melting of surface free fat also takes place in response to prolonged exposure to elevated storage temperatures. The melting point of surface free-fat was found to largely vary between −40 and 40 °C (Walstra and Jenness, 1984). After melting, fat sinks to particle interior in a form of encapsulated fat. Voids created by melting of fat are then occupied by surface active proteins. At 45 °C, all WPC

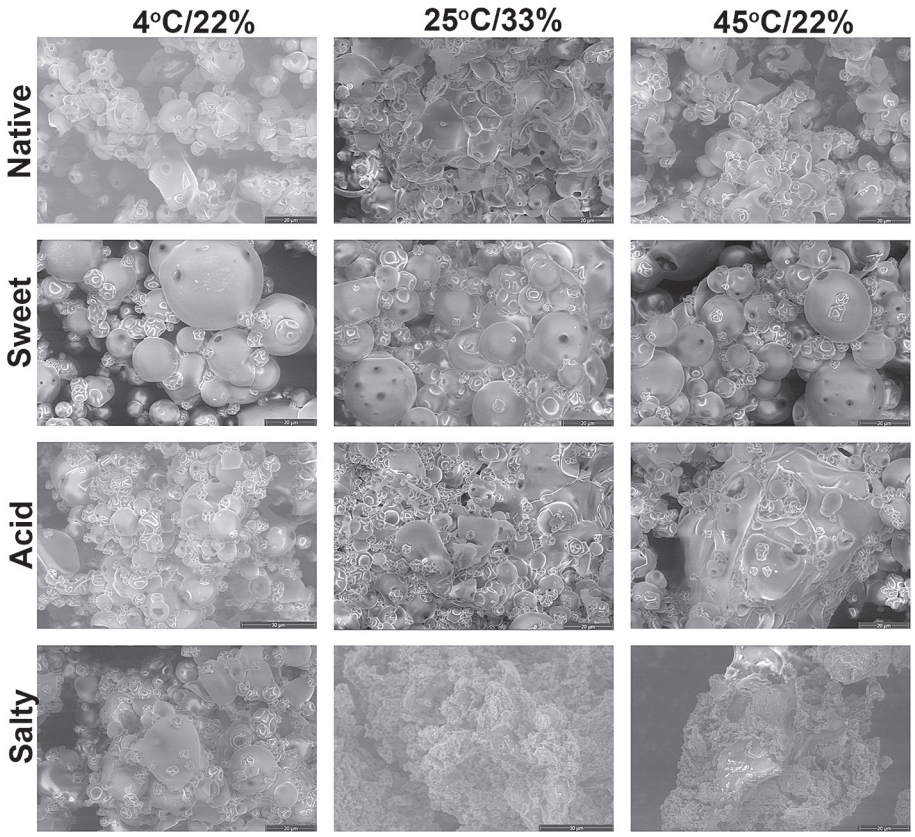

Fig. 2. Micrographs, obtained by scanning electron microscopy, of native, sweet, acid and salty-WPC powders equilibrated to 22 or 33% relative humidity at 4, 25 or 45 °C at the end of a 90-day storage period (90th day). The magnification was 3600x, with a bar indicating 20 μm length. The labelling of the columns denotes storage conditions including temperature (°C) and relative humidity (%), while the labelling of the rows depicts a type of the WPC powder.

demonstrated a similar trend - decline in surface fat coverage. Presence of fat on the WPC particle surface also promotes agglomeration by greater adhesion through hydrophobic interactions and small bridges of fat (Nijdam and Langrish, 2006). This was apparent in salty-WPC, which had high surface area covered by fat, in contrast to native-WPC which is predominantly covered by proteins. Although long hydrocarbon chains of fat are mainly responsible for the hydrophobicity of the particle surface, depending on the storage condition, hydrophobic amino acid (AA) side chains of proteins may also contribute to that. While all of the above reactions contribute to the changes in surface composition and related physical characteristics, in acid- and salty-WPC inherent compositional variations resulted in further differences.

Similar to native- and sweet-WPC, particle surface of acid-WPC was predominantly covered by proteins. At low temperature, i.e. 4 °C, protein denaturation was minimal in this powder as evidenced by reduction in particle size, high denaturation enthalpy and peak temperature, thus these proteins maintained their near native-state, with high surface activity. Although increase in protein coverage at this temperature is in line with surface activity and near-native state, it contradicts to preferential diffusivity (Kim et al., 2003) since proteins preferentially diffused to the surface over fat. The most likely reason is the hydrophilic core of acid-WPC, which is rich in minerals such as Ca. Inherent acidity and the severe heat treatment during manufacturing expose a great deal of hydrophobic AA residues to the protein surface (Nishanthi et al., 2017a). With high surface hydrophobicity, proteins tend to escape the hydrophilic core, gradually migrating towards the particle surface. In providing space for increasing protein concentration, some of surface free-fat sink to the particle interior. As proteins and fat migrated in counter-current directions at 4 °C, hydrophobicity of acid-WPC did not change significantly over the storage period. Further, 4 °C was found to exert minimal reaction rate and rate constant for reactivity of β-LG (Nishanthi et al., 2017b), thus confirming least conformational changes that expose interiorly located hydrophobic patches to the surface. From the beginning of storage, the particle size at 4 °C gradually declined further evidencing the minimal protein denaturation and their positioning as hydrophobic interactions are at their minimum below 25 °C and basically non-existent at 4 °C. In addition, decline in fat coverage on the particle surface might reduce the agglomeration, thus allow physical separation of the particles. In contrast, at 25 °C, surface protein content on acid-WPC significantly declined, providing space for excess fat deposition. Intensive protein aggregation occurred in acid-WPC throughout the storage period at 25 °C, as evidenced by the particle size increment and low denaturation enthalpy. As a result, some of the proteins present on the particle surface must have lost their surface activity, thus, replaced with fat, as confirmed by increasing fat coverage. At 25 °C, there were no evidence of protein migration from the core to the surface, mainly due to the increased reactivity of β-LG, forming salt bridges with Ca and aggregates with casein and other whey proteins present in the core, consequently acquiring a large molecular size, hence restricting the molecular mobility towards the surface. Significant increase of surface fat coverage at 25 °C might be a result of phase transition of lactose from amorphous to crystalline structure (Kelly et al., 2015), where crystal form of lactose can no longer retain fat, especially free-fat in a dispersed medium, therefore, release fat to the particle surface (Moreau and Rosenberg, 1993). While fat content increased on the surface at 25 °C, particle hydrophobicity decreased, implying

that the surface hydrophobicity under this condition was mainly governed by the proteins. Exposure of hydrophobic AA to the surface of proteins promotes particle aggregation through hydrophobic interactions, while high surface fat content cohesively binds particles together. At 45 °C, β-LG denature further exposing hydrophobic AA and oxidising free thiol groups, as evidenced by the conformational changes reported in our previous study (Nishanthi et al., 2017b). Denatured proteins present on the particle surface have low surface activity and hence could possibly be replaced by other compounds, mainly fat. This was not obvious in the current study, as coverage of proteins gradually increased with a concomitant decline of fat, which is due to melting of free-fat layer and migration into the interior (Walstra and Jenness, 1984; Kim et al., 2005b). The cause for migration of proteins from the core to the surface is yet unclear, as diffusivity of denatured proteins towards the surface is relatively low. Perhaps, highly hydrophilic core formed in abundance of Ca repel the proteins rich in hydrophobic AA, subsequently driving those towards the hydrophobic surface. From day 0, particles of acid-WPC were packed comparatively tighter than those in native and sweet-WPC. Particle aggregation was clearly temperature dependant resulting in large clusters by the end of storage, leaving only few particles in free-flowing state. Presence of lactose and lactic acid on the particle surface of acid-WPC attracts moisture during the storage, thereby, hydrating the particle surface enhancing molecular and particulate mobility and attractions.

Compared to other WPCs, 39% of particle surface of salty-WPC was covered by fat due to process induced fat solubilisation in the presence of high sodium concentration (Blaschek et al., 2007). At the same time, particle surface contained least protein coverage compared to other WPCs likely influenced by initial composition as the liquid concentrate contained only 19% of total protein (w/w) (Nishanthi et al., 2017a). Surface protein coverage did not vary significantly under most of the storage conditions, except at 25 °C and 33% RH, where protein coverage declined by 8%. Our previous study (Nishanthi et al., 2017b) revealed existence of intramolecular hydrogen bonds under 33% RH, suggesting greater presence of monomeric form of β-LG. Accordingly, presence of protein on the particle surface should have increased due to the high surface activity of near-native proteins. However, this was not obvious in the current study, as other components might have interfered with migration of proteins to the surface. Although surface fat and lactose content did not change significantly, mineral coverage increased by 8%, suggesting that minerals and proteins were moving in a counter-current flow at 25 °C and 33% RH. High osmotic pressure inside the particles and high humidity around the particle surface created a concentration gradient for Na, allowing a gradual migration of Na from the core to the surface to balance the osmotic gradient, hence, depositing more minerals on the surface and replacing proteins. In response to the increasing mineral coverage, hydrophobicity of the particle surface declined under these conditions. Compared to 25 °C, hydrophobicity of particle surface was high at 4 and 45 °C, with that at 4 °C as twice as that at 45 °C. No observable difference in the surface composition of salty-WPC at 4 and 45 °C was noted that could have caused the above difference in surface hydrophobicity. Most likely, the molecular mobility of Na and proteins is high at 45 °C, providing higher chance of interactions, mainly via salt bridges and electrostatic charge screening. These interactions consequently promote further aggregation, driven by hydrophobic interactions, thus reducing the amount of hydrophobic AA residues on the protein surface. In salty-WPC, cluster-like aggregate formation was prominent not only due to intensive protein aggregation but also due to cohesive forces occur between particle surfaces rich in fat (Kim et al., 2005a).

5. Conclusion

Compared to native- and sweet-WPC, origin and inherent composition of acid- and salty-WPCs greatly influenced changes of physical properties of the powders during storage, mainly surface composition of the powder particles. Similar to native- and sweet-WPC, particle surface of acid-WPC was covered mainly with proteins. In this powder, protein migration between the surface and the core was governed by mineral-rich particle core and its influence on denatured proteins. In salty-WPC, fat and minerals dominated the particle surface. Upon storage at 25 °C, proteins and minerals migrated in a counter-current flow in this powder, whereas, such a flow was not evident at either 4 or 45 °C. At the end of storage, cohesive arrangement of particles was common in both acid- and salty-WPC powders under all storage conditions, mainly due to protein aggregation and interparticle agglomeration through hydrophobic interactions and fat bridges. Among the four-WPCs studied, physical properties of acid- and salty-WPC behaved differently to that of native- and sweet-WPC, which might also differentiate their functional performances.

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Chapter 7: Impact of storage conditions on solubility, heat stability and emulsifying properties of selected spray dried whey protein concentrates

Chapter 7 presents the functional characteristics of stored sweet, acid and salty-whey protein concentrates powders at 25°C and two different relative humidity levels (22 and 33%) for a period of 90 days.

The paper entitles “Impact of storage conditions on solubility, heat stability and emulsifying properties of selected spray dried whey protein concentrates” by Manjula Nishanthi, Jayani Chandrapala and Todor Vasiljevic has been published in the peer-reviewed journal “LWT-Food Science and Technology” (2018), 92: 16 – 21. https://doi.org/10.1016/j.lwt.2018.01.068

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: Manjula Nishanthi Kottahachchi Kankanamge

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In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Manjula Kankanamge	80	Concept development, research question, hypothesis and manuscript preparation
Dr. Jayani Chandrapala	10	Concept development, research question, hypothesis and contribute in writing manuscript
Prof. Todor Vasiljevic	10	Concept development, statistical analysis, artwork preparation, contribute in writing manuscript and submission to journal

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

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Impact of storage conditions on solubility, heat stability and emulsifying properties of selected spray dried whey protein concentrates

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ABSTRACT

Storage conditions and composition of whey protein concentrates (WPCs) may destabilize the whey proteins (WPs) thus affect their functionality. Functional characteristics of native, sweet, acid and salty-WPC powders were analyzed after storage at 25 °C and RH (22, 33%) for 90 days. Native-, sweet- and acid-WPCs exhibited high solubility (82–97%), which was largely retained during storage. Solubility of salty-WPC was 52–55% initially, which gradually increased by ~5% during storage. Ionic sodium in salty-WPC interlinked WPs through salt bridges and charge screening, exposing reactive sites for intensive aggregation. Heat stability of salty-WPC was highest (64s), while lowest was recorded for native-WPC (16s). In presence of ionic sodium in salty-WPC, WPs denature due to salt bridging, charge screening and osmotic effects leading to intensive aggregation. High emulsion activity was recorded for salty-WPC, while those for other WPCs were similar. Emulsion stability varied as native- > acid- > salty- > sweet-WPC. High number of hydrophobic segments was likely exposed on the protein surface in salty-WPCs due to sodium-induced WP denaturation, thus increasing the number of proteins absorbed to emulsion interface, enhancing emulsion activity. Functionality of different WPCs during storage predominantly depended on the inherited composition.

1. Introduction

Whey proteins are widely used as ingredients in many food systems due to their unique nutritional qualities and functional characteristics (De Wit, 1990). Whey protein concentrates (WPC) and isolates (WPI) are mainly manufactured from sweet whey streams, released during the production of rennet type hard cheeses. These protein rich products are a popular choice of an ingredient in many products, such as energy supplements, nutritional drinks, protein bars, desserts, confectionaries and infant formula (Písecký, 2005). High protein and low lactose contents and the presence of fat and minerals at defined ratios provide a unique functional behavior, which can also be varied based on the solution characteristics such as temperature, ionic strength, pH and other compositional characteristics. Similar to sweet whey, acid and salty whey streams are generated in high volumes mainly due to increasing demand for strained yoghurts, cream and salted cheeses. Our previous study has shown that the WPCs produced out of these two whey streams could be excellent food ingredients due to their distinct protein characteristics and surface composition (Nishanthi, Chandrapala, & Vasiljevic, 2017b). Therefore, it would be desirable to study their functional characteristics to assess their applicability in food systems.

Solubility of WPCs is primarily important as it affects the applicability of whey proteins in foam, emulsion and gel systems. Whey proteins are globular in their native form with protein surfaces covered with hydrophilic residues thus are highly soluble over a broad range of pH (Zhu & Damodaran, 1994). However, their denaturation under varying environmental conditions including pH (Pelegrine & Gasparetto, 2005), temperature (Dissanayake & Vasiljevic, 2009), ionic strength, protein concentration (Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013a) and other compositional constituents including lactose, fat and organic acids (Dissanayake, Ramchandran, Piyadasa, & Vasiljevic, 2013b) expose hydrophobic and thiol groups, which were previously buried in the interior of the molecules, allowing for hydrophobic interactions, disulphide bonds and thiol/disulphide interchange reactions. This in turn results in poor rehydration but excellent heat stability (Dissanayake & Vasiljevic, 2009).

Whey proteins are also effective surface-active agents since they can lower interfacial tensions between hydrophobic and hydrophilic components in foods. Thus, WPC are widely used in the formation of oil-in-water and water-in-oil emulsions (McClements, 2009), due to stabilizing effect resulting from the formation of a protective barrier around the fat droplets, preventing emulsion coalescence. Emulsifying

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properties of whey proteins highly depend on the pH of the solution, as it determines the protein charge, solubility, flexibility and aggregation behavior (Das & Kinsella, 1989). At pH above isoelectric point (pH > 5), β-lactoglobulin (β-LG), the major whey protein, has high flexibility thus provides high stability against flocculation. It was suggested that for whey protein stabilized emulsions, conformational flexibility is more important than increased surface hydrophobicity (Shimizu, Kamiya, & Yamauchi, 1981). Heating (Demetriades, Coupland, & McClements, 1997b), ionic strength (Demetriades et al., 1997b), presence of sucrose (Kulmyrzaev, Bryant, & McClements, 2000), sodium ions (Demetriades, Coupland, & McClements, 1997a) or calcium ions (Ye & Singh, 2000) have been reported as factors that influence flocculation, thus stability of emulsions.

In addition to the environmental factors, storage of WPCs also alters the structure and interactions of whey proteins (Nishanthi, Chandrapala, & Vasiljevic, 2017c), thus affecting their functionality. For instance, storage of two WPCs with different protein concentrations (WPC34 and WPC80) revealed that change of solubility during 18 months of storage at 25, 30 and 35 °C and under 70 and 90% relative humidity (RH) was influenced by their protein concentration (Tunick et al., 2016). In the absence of compositional variations, storage time, temperature and RH influence the emulsification properties of WPC powders. For instance, Hsu and Fennema (1989) found that the storage temperature and humidity should not exceed 20 °C and 22%, respectively in order to largely retain the emulsification properties of WPC after 6 months of storage. Thus, storage conditions and composition of WPCs are important parameters that can influence combinedly functionality of whey proteins during storage (Norwood et al., 2017; Tunick et al., 2016).

Our previous studies on salty and acid whey streams revealed that their unique compositional features such as high contents of lactic acid and calcium (Ca) salts in acid whey and high content of sodium (Na) salts in salty whey impact whey proteins present in these whey streams and their concentrates by altering their structural characteristics and types of interactions, which are otherwise absent in native or sweet whey (Nishanthi, Vasiljevic, & Chandrapala, 2017a; Nishanthi et al., 2017b). In combination with compositional characteristics, storage conditions affected the protein conformation, their reactivity (Nishanthi et al., 2017c) and physical characteristics of the stored WPCs (Nishanthi, Chandrapala, & Vasiljevic, 2018). Storage-induced structural changes in proteins and other compositional constituents may alter the functionality of these WPCs. Thus, it is important to study the functional characteristics of these WPCs and their variation under different storage conditions. Therefore, the aim of the current study was to evaluate the solubility, heat stability and emulsifying property of sweet-, acid- and salty-WPC powders subjected to storage at 25 °C and two different relative humidities (RH) (22 and 33%) over a storage period of 90 days. Native-WPC was used as the reference.

2. Materials and methods

2.1. WPC production and storage

Four WPCs (native-WPC, sweet-WPC, acid-WPC and salty-WPC) were produced as described previously (Nishanthi et al., 2017b). The average composition of four WPCs on dry matter basis was: native-WPC – 841 g/kg protein and 40 g/kg lactose; sweet-WPC – 618 g/kg protein and 29 g/kg lactose; acid-WPC – 670 g/kg protein, 240 g/kg lactose, 70 g/kg lactic acid and 50 g/kg calcium (Ca); salty-WPC – 109 g/kg protein, 270 g/kg lactose and 120 g/kg sodium (Na). WPCs were dehydrated initially by placing them in vacuum desiccators over phosphorous pentoxide at room temperature (25 °C) for 4 weeks.

WPCs were stored at 25 °C and under two equilibrium relative humidities (RH; 22 and 33%) using saturated salt slurries containing potassium acetate (22 ± 2%) or magnesium chloride (33 ± 1%) respectively as described by Nishanthi et al. (2017c). Sampling was conducted at 3 time points representing 0, 30 and 90 days of storage. The first sampling (0 day) was conducted after allowing for a 2-week equilibration period from the initial date of the storage trial after pretesting for the water activity. After opening desiccators at a required time point, the analysis was carried out within 3 days.

2.2. Preparation of 50 g/kg protein dispersions from WPCs

Protein dispersions containing 50 g/kg of proteins were prepared by mixing the required amount of WPC powder in Milli-Q water at room temperature. The dispersions were stirred for about 2 h using a magnetic stirrer and stored in the refrigerator for overnight to ensure complete hydration. The pH of the whey protein dispersions did not change substantially throughout the storage trial. Thus, the average pH values for native-WPC, sweet-WPC, acid-WPC and salty-WPC were 6.5, 6.4, 4.2 and 5.5, respectively.

2.3. Solubility

Protein solubility was determined using a method developed by Morr et al. (1985). Ten mL of the prepared protein dispersion (50 g/kg of protein) was centrifuged (Avanti J-26XP centrifuge, Beckman Instrument Australia Pty Ltd., Gladesville, NSW, Australia) at 20,000 × g for 20 min at 20 °C. The supernatant was then filtered using a Whatman No. 1 filter paper. The protein content of the supernatant and the original dispersion was estimated using the Kjeldhal method (AOAC, 2000) and the nitrogen conversion factor of 6.38. Solubility was given by the protein content of the supernatant expressed as a percentage of the total protein content in the original dispersion.

2.4. Heat coagulation time (HCT)

Exactly 3 mL aliquots of 50 g/kg protein dispersions were placed in glass tubes (l0 mm diameter and 75 mm length), sealed and heated at 140 °C in a shaking oil bath (Ratek, Boronia, Australia). The heat exposure time until the first appearance of visible protein aggregates was considered as the HCT (Dissanayake, Liyanaarachchi, & Vasiljevic, 2012).

2.5. Emulsification

The method described by Dissanayake et al. (2012) based on Pearce and Kinsella (1978) was used to determine the emulsifying activity index (EAI) and emulsion stability index (ESI) to estimate the emulsification capacity and the stability of the emulsions formed of the dispersions prepared from WPCs, respectively. Approximately 8 mL of canola oil and 24 mL of 50 g/kg protein dispersions were mixed and held in a water bath heated to 40 °C for 20 min. The mixture was then homogenized using a B25 lab high shear mixer (Xingsheng Light Industry Machinery, Wenzhou, China) at 10,000 rpm for 1–2 min. Aliquots were obtained from the center of the emulsion samples for further analysis.

2.5.1. Emulsion activity index (EAI)

EAI was calculated as

$$EAI = \frac{2T}{\varphi\, C} \quad (1)$$

Where.

T – turbidity of the emulsion
Φ – oil volume fraction
C – weight of proteins per unit volume of aqueous phase prior to the formation of the emulsion

Oil volume fraction (φ) was calculated using equation (2), based on

the method described by Pearce and Kinsella (1978). Exactly 3 mL aliquots of the emulsions were dried in an oven at 120 °C overnight till a constant weight was obtained.

$$\varphi = \frac{C - A - E(B - C)}{C - A + (B - C)\left[\frac{(1+E)\cdot D_O}{Ds - E}\right]} \quad (2)$$

Where.

A – weight of empty pan
B – weight of the pan with the emulsion
C – weight of the pan with the dried emulsion
E – concentration of proteins in the dispersion (mass per unit mass of solvent)
D_0 – Density of the oil
Ds – Density of the protein dispersions

Turbidity (T) of the emulsion was determined using equation (3) (Pearce & Kinsella, 1978).

$$Turbidity\ (T) = 2.303\frac{A}{l} \quad (3)$$

Where.

A – absorbance of the diluted emulsion
^{l} – path length of the cuvette

2.5.2. Emulsion stability index (ESI)

Five mL of emulsion was refrigerated at 4 °C for 24 h. Absorbance of the diluted emulsions with 1 g/kg SDS was measured at 500 nm (Pearce & Kinsella, 1978). Turbidity prior (T) and after refrigeration was calculated using equation (2) and the change of turbidity (ΔT) was calculated with in the time interval (Δt) of 24 h.

$$ESI = \frac{T\ x\ \Delta t}{\Delta T} \quad (4)$$

2.5.3. Absorbed protein on the emulsion interface

The method described by Dissanayake et al. (2012) was used with minor modifications to estimate the content of adsorbed protein. The emulsions were centrifuged at 20 °C for 30 min at 12,000 × g (Avanti J-26XP centrifuge, Beckman Instrument Australia Pty Ltd., Gladesville, NSW, Australia). The Kjeldahl method with the nitrogen conversion factor of 6.38 was used to estimate the protein content of the liquid layer. Adsorbed protein was calculated as follows,

$$Absorbed\ protein\left(\frac{mg}{ml}\right) = Protein\ in\ dispersion\left(\frac{mg}{ml}\right) - Protein\ in\ aqueous\ layer\left(\frac{mg}{ml}\right) \quad (5)$$

2.6. Statistical analysis

The experiment was organized in a randomized split plot blocked design with 2 main factors - a type of WPC as the main plot and RH as the subplot. The replications served as blocks. Replicated samples were sub sampled and analyzed. The results were statistically analyzed using the General Linear Model (GLM) and ANOVA procedure. The level of significance was preset at $p < 0.05$.

3. Results

3.1. Solubility of WPCs

Solubility of WPCs was significantly ($p < 0.05$) affected by the type of WPC and the humidity during storage (Table 1), while storage time had no major impact. At the beginning of the storage, native-WPC showed the highest solubility among all four WPCs, while salty-WPC had a significantly ($p < 0.05$) low protein solubility under both RH levels. During 90 days of storage, solubility of native-, sweet- and acid-WPCs did not change significantly. Solubility of native-, sweet- and salty-WPCs was high under 22%, as compared to 33% RH. On the contrary, acid-WPC had a greater solubility under 33 than that at 22% RH.

Table 1
Solubility (%) and heat coagulation time (HCT) (s) of whey protein concentrates (WPCs) stored at 25 °C and relative humidity of 22 or 33% for a storage period of 90-days.

Sample	Humidity (%)	Storage period (days)	Solubility (%)	HCT (s)
Acid-WPC	22	0	91.2^{a}	69^{a}
		30	90.1^{ah}	53^{b}
		90	88.9^{ahl}	37^{c}
	33	0	94.0^{b}	81^{d}
		30	90.2^{ah}	52^{b}
		90	89.6^{ahl}	51^{b}
Native-WPC	22	0	97.2^{c}	55^{b}
		30	93.2^{a}	20^{ef}
		90	90.2^{ah}	16^{e}
	33	0	92.6^{a}	43^{c}
		30	92.0^{a}	22^{f}
		90	90.2^{ah}	16^{e}
Salty-WPC	22	0	55.7^{d}	98^{g}
		30	58.6^{e}	72^{a}
		90	61.5^{f}	54^{b}
	33	0	52.3^{g}	92^{h}
		30	55.2^{d}	88^{h}
		90	57.7^{de}	73^{a}
Sweet-WPC	22	0	91.2^{a}	53^{b}
		30	87.9^{hk}	32^{ci}
		90	85.0^{i}	28^{i}
	33	0	81.7^{j}	54^{b}
		30	85.4^{ik}	27^{fi}
		90	86.8^{ikl}	27^{fi}
SEM			0.9	2

$^{a\text{-}l}$ Different lowercase superscripts in the same column depicts the significant differences between means of solubility and heat coagulation time (HCT). Results are expressed as means of two independent replications. SEM = Pooled standard error of the mean.

3.2. Heat stability of WPCs

Heat stability of WPCs was measured as the heat coagulation time (HCT) and presented in Table 1. Storage time, RH and the composition of WPC significantly ($p < 0.05$) influenced the HCT. At all times, under any RH level, HCT varied in the order of salty- > acid- > sweet- > native-WPC. In general, HCT declined throughout the storage irrespective of RH. Heat stability of native-WPC declined greatly (71 and 62% under 22 and 33%, respectively), while that of salty-WPC declined to a lesser extent (45 and 20% under 22 and 33%, respectively).

3.3. Emulsification properties of WPCs

The type of WPC and the storage time significantly ($p < 0.05$) influenced the emulsion activity index (EAI), while relative humidity had only a minor influence (Table 1). Salty-WPC showed significant effect on EAI with storage time, while other WPCs had no significant effect. EAI of native-, sweet- and acid-WPCs were approximately similar at the start of the storage. Under all storage conditions, EAI decreased over 90 days with averages of 1241, 783, 886 and 7671 m^2/g for native-, sweet-, acid- and salty-WPCs, respectively.

Emulsion stability index (ESI) of WPCs stored at 25 °C and RH levels of 22 and 33% for 90 days are shown in Table 1. ESI of WPC was significantly ($p < 0.05$) influenced by all experimental factors including storage time, humidity and the type of WPC. In all WPCs, ESI observed at the beginning of the storage was significantly ($p < 0.05$) greater

Table 2
Emulsion activity index (EAI), emulsion stability index (ESI) and absorbed proteins (mg/mL) of emulsions prepared from whey protein concentrates (WPCs) stored at 25 °C and relative humidity of 22 or 33% for a storage period of 90-days.

Sample	Relative humidity (%)	Storage period (days)	EAI (m^2/g)	ESI (h)	Absorbed protein (mg/mL)
Native-WPC	22	0	2336^{a}	98^{d}	4^{a}
		30	1709^{a}	44^{bf}	5^{a}
		90	1185^{a}	35^{b}	10^{b}
	33	0	2325^{a}	233^{e}	4^{a}
		30	1179^{a}	67^{afl}	4^{a}
		90	994^{a}	37^{bi}	9^{a}
Sweet-WPC	22	0	2523^{a}	55^{fijm}	5^{a}
		30	2332^{a}	43^{bj}	6^{a}
		90	1742^{a}	40^{bj}	17^{e}
	33	0	2983^{a}	140^{k}	5^{a}
		30	2867^{a}	50^{bjl}	6^{a}
		90	2198^{a}	46^{bm}	16^{ef}
Acid-WPC	22	0	2483^{a}	76^{a}	3^{a}
		30	1713^{a}	50^{bf}	4^{a}
		90	1469^{a}	41^{bf}	11^{b}
	33	0	2278^{a}	213^{c}	4^{a}
		30	1707^{a}	45^{bf}	5^{a}
		90	1519^{a}	39^{bf}	11^{bf}
Salty-WPC	22	0	18789^{b}	75^{ai}	3^{a}
		30	15815^{cf}	65^{ahilm}	3^{a}
		90	11787^{d}	44^{bj}	42^{c}
	33	0	16545^{ec}	172^{g}	2^{a}
		30	14412^{f}	81^{da}	2^{a}
		90	8204^{g}	46^{bhj}	34^{d}
SEM			678	7	2

[a-m] Different lowercase superscripts in the same column depicts the significant differences between means of emulsion activity index, emulsion stability index and absorbed proteins. Results are expressed as means of two independent replications. SEM = Pooled standard error of the mean.

under 33% RH, than that under 22% RH. ESI varied as native- > acid- > salty- > sweet-WPC at the beginning of storage, irrespective of the RH conditions. ESI declined with increase in storage time under both RH levels.

Amount of adsorbed proteins (mg/mL) at the interface in the emulsions prepared from each WPC stored under 22% and 33% RH at 25 °C is shown in Table 2. Storage time and the type of WPC significantly ($p < 0.05$) influenced the variation of the amount of adsorbed proteins. At the beginning of the storage, the amount of adsorbed proteins was approximately similar for all WPCs. However, it increased under all storage conditions by 5.8, 11.8, 7.6 and 35.4 mg/mL in native, sweet, acid and salty-WPC, respectively, during 90 days. By the end of the storage, salty-WPC showed significantly high levels of adsorbed proteins, followed by sweet- and acid-WPC, while native-WPC showed the least.

4. Discussion

4.1. Solubility

Results of the current study highlight how compositional variations of WPCs influenced their solubility during storage. In this case, the relative humidity of storage exhibited a significant impact on the solubility while storage time showed very minor influence. In addition, native-, sweet- and acid-WPCs exhibited a good solubility immediately after manufacturing, which was largely retained for the 90 days storage period at 25 °C. On contrary, salty-WPC was the least soluble. Greater coverage of particle surface with the native-form of proteins (Nishanthi et al., 2017c) allow greater extent of protein-water interactions, thus resulting in a high solubility of native-WPC. Proteins in salty-WPC are subjected to charge screening in the presence of high concentration of positively charged sodium ions (Zhu & Damodaran, 1994). In addition, the high osmotic pressure surrounding the proteins exerted in presence of high concentrations of sodium ions unfolds the tertiary structures, exposing reactive sites, such as free thiol groups and hydrophobic segments for protein-protein interactions (Nishanthi et al., 2017b). Aggregated proteins have unevenly distributed charge on the protein surface with comparatively less hydrophilic segments, thus limiting the number of protein-water interactions. Furthermore, the surface of the salty-WPCs is primarily covered with fat (~40% of surface area) rendering the particle surface more hydrophobic (Nishanthi et al., 2017c), hence promoting particle agglomeration and reducing solubility. Opposing effect can be expected in presence of high amount of minerals on the surface of salty-WPCs (Nishanthi et al., 2017c), which would enhance hydrophilic interactions with water, and thereby increase solubility. However, the results of the current study suggest that protein denaturation, subsequent aggregation and high surface fat coverage govern the solubility of salty-WPC. Unlike the other WPCs, solubility of salty-WPC increased over the storage period. The increased amounts of lactose and minerals on the surface and decreased surface fat of salty-WPCs (Nishanthi et al., 2017c) increased the solubility during 90 days of storage, irrespective of RH. The exposure of more hydrophilic segments on the particle surface forms a saturated monolayer surrounding the particle surface with the increased absorption of water (Kelly, O'Mahony, Kelly, & O'Callaghan, 2016). The solidified salts on the particle surface dissolve, which limits their osmotic impact on the proteins. This in turn lowers the extent of protein aggregation and thereby increases the protein solubility. The relative humidity influenced the solubility of WPCs in a complex manner, as indicated by increased solubility for native, sweet and salty WPCs and a decline in solubility for acid-WPC under 22% RH as compared to 33%. Compositionally, acid-WPC consists of high concentrations of calcium based lactate salts and lactic acid. Due to presence of lactic acid, acid WPC had a pH of 4.2. Due to the low dissociation constant of lactic acid, deprotonation takes place readily. This in turn lowers the negative surface charge density of whey proteins, thus inducing protein aggregation through non-covalent interactions such as van der Waals and electrostatic attractions (Dissanayake et al., 2013b). Ca also promotes interlinking of proteins through formation of ionic bridges and shielding of repulsion (Zhu & Damodaran, 1994). In addition, Ca binds to strong binding sites of whey proteins and induces conformational changes. These conformational changes expose the hydrophobic segments on the protein surface, facilitating the protein-protein interactions and aggregation (Zhu & Damodaran, 1994). Thus, low relative humidity increases the impact of Ca and lactic acid resulting in a decreased solubility.

4.2. Heat stability

Heat stability is an important functional property, which demonstrates the ability of whey proteins to endure commercial heat processing, without subjecting them to detrimental physical changes such as precipitation, turbidity, gelation, increments in viscosity and phase separation. Heat stability of WPC depends on its composition, mainly whey protein profile and non-protein components such as sugars, salts and fat. Among two main whey proteins, thermal stability of α-lactalbumin (α-LA) is greater than that of β-LG, mainly due to lack of free thiol groups and abundance of holo-form of α-LA, in which Ca is bound to its binding site (McSweeney & Fox, 2013). Throughout the storage, HCT varied in the following order: salty- > acid- > sweet- > native-WPC. High heat stability of salty-WPC could be mainly attributed to the presence of whey proteins in an intensively aggregated form, thus with no available reactive sites such as free thiol groups for further aggregation. Electrostatic charge screening and salt bridge formation induced by the presence of high concentrations of Na reduce the intermolecular distance between proteins allowing for covalent bonding. Similar to salty-WPC, heat destabilization of acid-WPC is due to the covalent interlinking of proteins in presence of high Ca concentration

(Crowley et al., 2014). Furthermore, high lactic acid concentration, thus low pH, leads to a high degree of protein aggregation due to prevalent positive charge, thus creation of more non-covalent bonds such as hydrophobic, van der Waals and electrostatic interactions. Acid-WPC is comparatively high in casein, thus high number of denatured whey proteins are in association with the caseins, providing a stability against heat (Singh, 2004). In contrast, in sweet-WPC, concentration of ionic salts is low comparative to salty- and acid-WPC, thus preventing protein interlinking through covalent and non-covalent interactions. Therefore, most of the proteins are in non-aggregated, reactive state, thus less heat stabile. The lowest heat stability of native-WPC is attributed to the presence of large amounts of native whey proteins due to the minimal thermal treatment during its manufacturing process (Nishanthi et al., 2017b). Undenatured whey proteins are easily susceptible to unfolding and aggregation at 140 °C. Native whey proteins present in this WPC are more prone to conformational changes exposing reactive thiol and hydrophobic sites enabling various reactions in comparison to aggregated whey proteins present in salty-WPC (Nishanthi et al., 2017b).

4.3. Emulsification properties of WPCs

Emulsion activity index (EAI), as one of two important emulsification parameters, is governed by capacity of a protein to unfold and adsorbed over the interface, stabilizing newly created area (Pearce & Kinsella, 1978). Consequently, molecular flexibility and conformational changes of proteins are extremely important, as these govern other properties such as surface hydrophobicity, protein flexibility, solubility, a degree of disulphide bonds, affinity of proteins for the oil–water interface, a level of hydrogen interactions and other stabilizing forces (Monahan, McClements, & Kinsella, 1993). Slight denaturation improves the emulsifying properties by exposing hidden hydrophobic groups, thus balancing the hydrophilic and hydrophobic interactions to minimize the surface tension (Dissanayake et al., 2012). Emulsion stability index (ESI) reflects the capacity of emulsion droplets to remain dispersed without separation by creaming, coalescing, and flocculation. Main contributory factor is the consistency of the interface, which does not change with time (Dissanayake & Vasiljevic, 2009). Emulsions with increased net negative charge exert adequate repulsion that enables dispersion of droplets and retards coalescence (Klemaszewski & Kinsella, 1991). Salty-WPC had the highest EAI, which could be attributed to the presence of high concentrations of Na ions. Sodium ions enhance charge screening, exposing the interiorly located hydrophobic segments of β-LG such as Cys_{121}-Val_{123}, Ala_{81}-Phe_{83}, Ile_{71}-Ala_{73} and Leu_{103}-Phe_{105}. Due to greater exposure of hydrophobic amino acids, whey proteins have a considerable potential to engage in hydrophobic interactions with the oil phase and stabilize emulsions. Such exposure of previously buried hydrophobic sites is further favored by enhanced osmolality in the presence of elevated Na concentration. In addition to proteins, fat present on the surface of salty-WPC plays an important role in emulsion activity. Coverage of free fat is high on the particle surface of salty-WPC (Nishanthi et al., 2017c), which contains a hydrophobic hydrocarbon chain and hydrophilic carboxyl group, thus a high affinity to oil-water interface. Furthermore, the amount of adsorbed proteins to the oil-water interface was highest in the emulsion produced with salty-WPC at the end of storage, implying a high EAI. Similar to Na in salty-WPC, Ca in acid-WPC interacts with whey proteins through two possible mechanisms. First, Ca screens the negative charge repulsion of proteins to a greater extent, reducing the intermolecular distance, thus allowing protein aggregation via intermolecular non-covalent interactions. Second, Ca crosslinks proteins via salt bridges (Zhu & Damodaran, 1994), thus exposing reactive sites to the protein surface, consequently imparting a thermodynamically more favorable condition for a stable emulsion. Furthermore, inherent acidity of acid-WPC with lactic acid imparts a prevailing positive charge on whey protein surface, misbalancing the inter-protein electrostatic environment. This in turn exposes more hydrophobic sites to the surface, resulting in a comparatively high EAI (Dissanayake et al., 2012). With the influence of Ca and lactic acid, comparatively high EAI is expected to occur in acid-WPC, compared to native- and sweet-WPCs, which was not apparent in the current study. Perhaps, the intensive heat treatment applied in the manufacturing of Greek yoghurt, from which this acid whey was collected for the current study, caused an excessive denaturation and aggregation, consequently decreasing the affinity between proteins and the dispersed phase (de la Fuente, Singh, & Hemar, 2002). This is confirmed by a very low amount of adsorbed proteins onto the oil-water interface in the emulsion prepared from acid-WPC. In contrast, emulsion stability of acid- or salty-WPCs was lower compared to that of native-WPC, mainly due to inherently high concentrations of Na in salty-WPC and lactic acid or Ca in acid-WPC. High concentration of Ca in acid-WPC promotes the formation of insoluble aggregates between denatured whey proteins. Denatured and aggregated whey proteins have a localized and unevenly distributed negative charge, thus have low capacity to maintain the repulsion between emulsion droplets. Similar effect can be seen in salty-WPC due to the Na-induced protein aggregation. Furthermore, Ca and Na in the dispersed medium of emulsions formed by acid- and salty-WPCs form bridges between droplets and promote flocculation, consequently destabilizing the emulsion. The salts present in the dispersed medium partially shield the electrostatic repulsive forces, thus allow droplet flocculation through hydrophobic interactions and thiol-disulphide interchange reactions of proteins present in different droplets. This explains why emulsion stability of salty-WPC was low despite of its emulsion activity being high. In contrast to salty- and acid-WPC, native-WPC formed the most stable emulsions, mainly attributed to a minimal protein denaturation occurring during its manufacturing process. As shown by the high kinetic energy and low reaction order of β-LG reactivity (Nishanthi et al., 2017c), whey proteins in native-WPCs are in their native form, with a high overall negative charge, consequently preventing close approach of droplets and stabilizing the emulsion. In native-WPC, high solubility supports formation of a stable emulsion through rapid adsorption at the interface to form a strong cohesive film. In the current study, the emulsion prepared using sweet-WPC was the least stabile, most likely due to presence of high concentration of Maillard reaction products (Norwood et al., 2016). Sweet-WPC contained 29 g/kg of lactose, which is adequate enough to conjugate with β-LG and α-LA (Nishanthi et al., 2017c), consequently reducing their surface activity. When surface active proteins are insufficient to fully cover the newly formed oil-water interface, flocculation may occur gradually, disturbing the stability of the emulsion. In all WPCs, EAI and ESI declined as storage progressed, mainly due to gradual progression of whey protein aggregation during storage. As a result of denaturation and aggregation related structural changes, molecular flexibility is minimized decreasing the affinity of whey proteins for the oil-water interface, thus affecting the emulsion activity. In addition, the negative charge distribution becomes localized on the protein surface, thus affecting the emulsion stability. Exposed hydrophobic and thiol segments in all types of emulsions lead to a greater amount of proteins to be adsorbed onto the emulsion interface, thus increasing the proteins accumulation in oil-water interface.

5. Conclusion

Functionality of different WPCs varied based on their inherent composition and storage conditions. Protein conformational changes occur due to the combined effect of storage and compositional variation governing functional properties among different WPCs. Mainly the high concentration of sodium salts in salty-WPC caused whey protein denaturation and aggregation, thus leading to low solubility and high heat stability. Denatured whey proteins and high fat coverage on particle surface of salty-WPC led to high emulsification activity, but low emulsion stability, compared to the reference native-WPC. Therefore, salty-WPC could potentially be used in heat-processed emulsions such

as beverages, processed cheese spreads and salad dressings with minor modifications to mediate its salty flavor. Furthermore, presence of lactic acid in high concentration led acid-WPC to be equally soluble to native-WPC. The protein interlinking in presence of calcium in acid-WPC, subsequently resulted in intensive aggregation, thus provide a comparatively high heat stability. For these reasons, the acid-WPC may have potential applications in infant formula, desserts and bakery products.

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Chapter 8: Conclusions and future directions

8.1 Overall conclusions

In commercially derived whey streams, compositional variations and applied processing methods impact the properties of the whey proteins (WPs). The pH, the change in ionic strength of acid whey and the applied heat treatment during the generation of acid whey induce conformational changes in the WPs and facilitate aggregate formation. The WPs present in acid whey aggregated via hydrophobic interactions and covalent linkages. Both thiol/disulphide mediated reactions and non-thiol/disulphide reactions appeared to be responsible for aggregate formation via covalent interactions in acid whey. Aggregation of WPs in acid whey was characterized by intermolecular β-sheet aggregation and intermolecular β-sheet cross linking and most importantly, had a less influence on the secondary protein structure. In contrast, WPs aggregated via strong covalent bonds and weak van der Waals, hydrogen and electrostatic interactions, exposing the hydrophobic sites to the surface in salty whey. The covalent aggregates were created by both thiol/disulphide mediated and non-thiol/disulphide mediated reactions and were characterized by intermolecular β-sheet aggregation and loss of other native secondary structure descriptors.

Aforementioned unique properties of WPs in different whey streams due to inherent compositional differences further changed during processing, especially during ultrafiltration and spray drying. Changes occur during processing are clearly governed by the type of whey stream. Liquid whey protein concentrates (WPCs) of acid and salty whey were characterized with aggregated proteins, created by covalent crosslinking among individual WPs (β-LG, α-LA, BSA and LF) and/or caseins, which transpired mainly in response to compositional differences (high acidity and salinity) and heat treatments such as pasteurization and

evaporation applied during their manufacturing processes. During spray drying of native and sweet-WPC, protein aggregation and related physical characteristics were conveyed from the liquid WPC to the spray dried WPC powders. However, transfer of protein characteristics is less likely to occur in acid and salty whey streams due to the characteristic changes associated with spray drying of liquid acid- and salty-WPC. During spray drying of acid-WPC, Maillard reaction products are formed by crosslinking and polymerization of WPs. In the absence of moisture in salty-WPC powder, Na salt crystallized, reversing the covalent bonding between lactoferrin (LF) molecules. Therefore, it was clearly evident that the compositional, surface and particle organization characteristics of WPC powders present a cumulative effect of those properties of original feed stream, concentrate, and changes induced by the spray drying.

In addition to the original composition and further processing, storage conditions clearly affected the molecular level properties of WPC powders obtained from different whey streams. Protein aggregation occurred predominantly through covalent crosslinking under all storage temperatures (4, 25 and 45°C), while higher temperatures accelerated aggregation. Relative Humidity (RH) levels (22 and 33%) had a variable effect on the protein aggregation, mainly based on the type of WPC powder. Disulphide and non-disulphide covalent crosslinking of β-LG was the main aggregation pathway in all WPC powders, whereas, involvement of α-LA, BSA and caseins depended on the type of WPC powder. In parallel to the protein aggregation, disappearance of β-LG followed a first-order reaction in native- and sweet-WPC powders, while it was closely a second-order reaction in acid- and salty-WPC powders. Compositional variations associated with origin of acid- and salty-WPC powders facilitated the β-LG loss during storage.

The surface composition of the powders was greatly influenced by storage and was varied depending on the whey stream. Similar to native- and sweet-WPC powders, the particle surface of acid-WPC powder was covered mainly with proteins. The protein migration between the surface and the core was governed by the mineral-rich particle core and its influence on denatured proteins present in acid whey. In salty-WPC powder, fat and minerals dominated the particle surface. Upon storage at 25°C, proteins and minerals migrated in a counter-current flow in this powder, whereas, such a flow was not evident at either 4 or 45°C. At the end of storage, cohesive arrangement of particles was common in both acid- and salty-WPC powders under all storage conditions, mainly due to protein aggregation and inter-particle agglomeration through hydrophobic interactions and fat bridges. Among the four-WPC powders studied, physical properties of acid- and salty-WPC powder behaved differently to that of native- and sweet-WPC powder, which might also differentiate their functional performances.

Functionality of different WPC powders varied based on their inherent composition and storage conditions. Protein conformational changes occur due to the combined effect of storage and compositional variation governed the functional properties among different WPC powders. Mainly the high concentration of sodium salts in salty-WPC powder caused WP denaturation and aggregation, thus leading to low solubility and high heat stability. Denatured WPs and high fat coverage on particle surface of salty-WPC powder led to high emulsification activity, but low emulsion stability, compared to the reference native-WPC powder. Therefore, salty-WPC powder could potentially be used in heat-processed emulsions such as beverages, processed cheese spreads and salad dressings with minor modifications to mediate its salty flavor. On the other hand, presence of lactic acid (LA) in high concentration

led acid-WPC powder to be equally soluble to native-WPC powder. The protein interlinking in presence of calcium in acid-WPC powder, subsequently resulted in intensive aggregation, thus provide a comparatively high heat stability. For these reasons, the acid-WPC powder may have potential applications in infant formula, desserts and bakery products.

Overall, salty- and acid-WPC powders can be commercially viable food ingredients similar to sweet-WPC powder. However, their inherent composition has a great influence on the properties of WPs present. These unique compositional characteristics influence the ultrafiltration and spray drying, the two most important steps in downstream processing and also during storage. Different additional processing approaches appear to be required to convert salty and acid whey to functionally viable whey streams similar to sweet whey. Demineralization may be needed for salty whey to remove the salts, while nanofiltration can be incorporated for acid whey to remove LA. These two processing approaches may adjust acid and salty whey streams more compositionally comparable with sweet whey, thus enabling production of whey powders with similar functionalities as sweet whey powder. Fundamental knowledge generated in this study may facilitate the re-utilization of acid and salty whey streams through composition-adjusted ultrafiltration and spray drying to manufacture whey powders that could be potentially used in various food systems.

8.2 Future directions

The findings of the current research have revealed that mediation of unique compositional differences is required for acid and salty whey streams. Although feasible industrial approaches are available such as demineralization to minimize effect of minerals and nanofiltration to reduce the content of LA, compositional differences affect progression of these steps, extending the processing time. Therefore, future investigations should be

devised to assess how processing delays associated with the unique composition of these two whey streams can be overcome, therefore achieving effective downstream processing methods.

Lactosylation occurred during the storage of WPC powders. The occurrence of lactosylation can deteriorate the sensory and nutritional quality of WPC powders, converting them to unsaleable products. Therefore, establishing more fundamental knowledge on the degree of lactosylation during storage, could facilitate the establishment of optimal storage conditions.

The current study mainly focused on evaluating the performances of WPCs in emulsifying, solubilizing and heat stability. Further investigation to analyze their gel forming ability would be important in many industrial applications. Especially, the properties of cold-set gels, as affected by the storage conditions, would be essential to study in order to optimize the textural and sensory characteristics of these gels.

Further, the denaturation and hydrolysis processes of WPs present in acid and salty-WPC powders may be different inside the gut compared to those of sweet and native-WPC powders. Therefore, *in vitro* and *in vivo* studies on the peptide release from WPs present in these two WPC powders would be another direction of future research to identify the nutritional benefits of these WPCs. In overall, the listed recommendations for future work could broaden the applicability of these newly created food ingredients.