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Ollscoil na hÉireann, Corcaigh  
**The National University of Ireland, Cork**  
**University College Cork**

**School of Food and Nutritional Sciences**

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**Preparation, characterisation and  
functional applications of whey protein-  
carbohydrate conjugates as food  
ingredients**

*Thesis presented by*

**Eve M. Mulcahy**

*for the degree of*

**Doctor of Philosophy**

*in*

**Food Science and Technology**

*May 2017*

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# Declaration

I hereby declare that the work submitted is entirely my own and has not been submitted to any other university or higher education institute, or for any other academic award in this university.

Signature:

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Eve Mulcahy

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Date

*This thesis is dedicated to my parents,*

*Rose and Billy*

## Abstract

The core technological hurdles limiting the use of whey protein ingredients in value-added nutritional beverages include poor solubility, heat stability and lack of clarity, particularly in high-acid or high ionic strength, ready-to-drink systems such as those found in sports and performance nutritional products. The studies in this thesis detail novel research into production, characterisation and functional properties of whey proteins/peptides conjugated with a range of starch hydrolysis products. Targeted structural modification of the components, e.g., hydrolysis of the whey proteins, or increasing dextrose equivalent value of the starch hydrolysis products, increased the rate of protein-carbohydrate conjugation on wet heating (initial pH 8.2, 90°C). Effective conjugation, with limited associated progression of the Maillard reaction into the advanced stages, was achieved at 8 h of wet heating. The heating mode also influenced the rate and extent of conjugation; an equivalent level of conjugation was achieved on dry heating of WPI with maltodextrin (MD) under milder conditions (60°C, 79% relative humidity, for 24 h) compared to wet heating for 8 h. Solutions of conjugated whey proteins/peptides had improved protein solubility and thermal stability compared to the respective unconjugated control solutions; this was attributed to the covalent attachment of starch hydrolysis products to the proteins/peptides. The choice of starch hydrolysis product (i.e., MD or corn syrup solids) also influenced the functionality of the conjugated proteins/peptides. Dry heating of WPI with MD maintained the native structure of the whey proteins while increasing both the protein solubility and thermal stability, compared to untreated whey protein. The influence of aggregation state on availability of amino groups in whey proteins was investigated to gain a better understanding of the ability of whey proteins to undergo conjugation with carbohydrates. Sulfhydryl-disulphide interchange and disulphide-bond mediated aggregation of whey proteins resulted in decreased availability of amino groups as measured by the *o*-phthalaldehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods due to the amino groups being located within large, dense protein aggregates. Overall, the findings presented in this thesis constitute a significant advancement of the body of knowledge on whey protein/peptide-carbohydrate conjugate ingredients, specifically in terms of optimising their preparation and studying their functional characteristics to facilitate their incorporation into food formulations.

## **Publications**

### **Publications in peer-reviewed, international, scientific journals:**

**Mulcahy, E. M.**, Mulvihill, D. M., & O'Mahony, J. A. (2016). Physicochemical properties of whey protein conjugated with starch hydrolysis products of different dextrose equivalent values. *International Dairy Journal*, 53, 20-28. (Based on Chapters 3 and 4)

**Mulcahy, E. M.**, Park, C. W., Drake, M., Mulvihill, D. M., & O'Mahony, J. A. (2016). Improvement of the functional properties of whey protein hydrolysate by conjugation with maltodextrin. *International Dairy Journal*, 60, 47-54. (Based on Chapter 6)

**Mulcahy, E. M.**, Park, C. W., Drake, M., Mulvihill, D. M., & O'Mahony, J. A. (2017). Enhancement of the functional properties of whey protein by conjugation with maltodextrin under dry heating conditions. *International Journal of Dairy Technology*. (In press, based on Chapter 5)

**Mulcahy, E. M.**, Fargier-Lagrange, M., Mulvihill, D. M., & O'Mahony, J. A. (2017). Characterisation of heat-induced protein aggregation in whey protein isolate and the influence of aggregation on the availability of amino groups as measured by the *o*-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods. *Food Chemistry*. (In press, based on Chapter 2)

O'Mahony, J. A., Drapala, K. P., **Mulcahy, E. M.**, & Mulvihill, D. M. (2016). Controlled glycation of milk proteins and peptides: Functional properties. *International Dairy Journal*. (In press, based, in part, on Chapter 1)

## **Oral Presentations**

**Mulcahy, E. M.**, Mulvihill, D. M. & O'Mahony J. A. (2014). Functional properties of whey protein-maltodextrin conjugates prepared by wet and dry heating methods, 7<sup>th</sup> *International Whey Conference, Rotterdam, The Netherlands, 7-9<sup>th</sup> September 2014, 1<sup>st</sup> place 'Young Scientist Competition*.

**Mulcahy, E. M.**, Mulvihill, D.M. & O'Mahony J.A. (2014). Selected functional properties of whey protein-maltodextrin conjugates prepared by wet and dry

heating methods, *43<sup>rd</sup> Annual Food Research Conference, UCD, Dublin, Ireland, 10-11<sup>th</sup> December 2014.*

**Mulcahy, E. M.,** Mulvihill, D. M. & O'Mahony J. A. (2015). Improvement of the physicochemical and functional properties of whey protein hydrolysates by conjugation, *American Dairy Science Association (ADSA) Annual Meeting, Florida, USA, 12-16<sup>th</sup> July 2015, 1<sup>st</sup> place Dairy Research Institute Graduate Student Paper Presentation Contest.*

**Mulcahy, E. M.,** Mulvihill, D. M. & O'Mahony J. A. (2015). Improvement of the physicochemical and functional properties of whey protein hydrolysates by conjugation, *9<sup>th</sup> NIZO Dairy Conference, Milk Protein Functionality, Papendal, The Netherlands, 30<sup>th</sup> September–2<sup>nd</sup> October 2015.*

## **Poster Presentations**

**Mulcahy, E. M.,** Mulvihill, D. M. & O'Mahony J. A. (2013). Conjugation of whey protein with different starch hydrolysis products through the Maillard reaction. *42<sup>nd</sup> Annual Food Research Conference, Teagasc Food Research Centre, Ashtown, Dublin, Ireland, 27-28<sup>th</sup> June 2013.*

**Mulcahy, E. M.,** Mulvihill, D. M. & O'Mahony J. A. (2016). Improvement of functional properties of whey protein hydrolysate by conjugation with maltodextrin. *IDF Dairy Science & Technology Symposia 2016, Dublin, Ireland, 11-13<sup>th</sup> April 2016.*



# Chapter 1

## *Review of relevant literature on milk proteins and Maillard-induced conjugation of milk proteins with carbohydrates*

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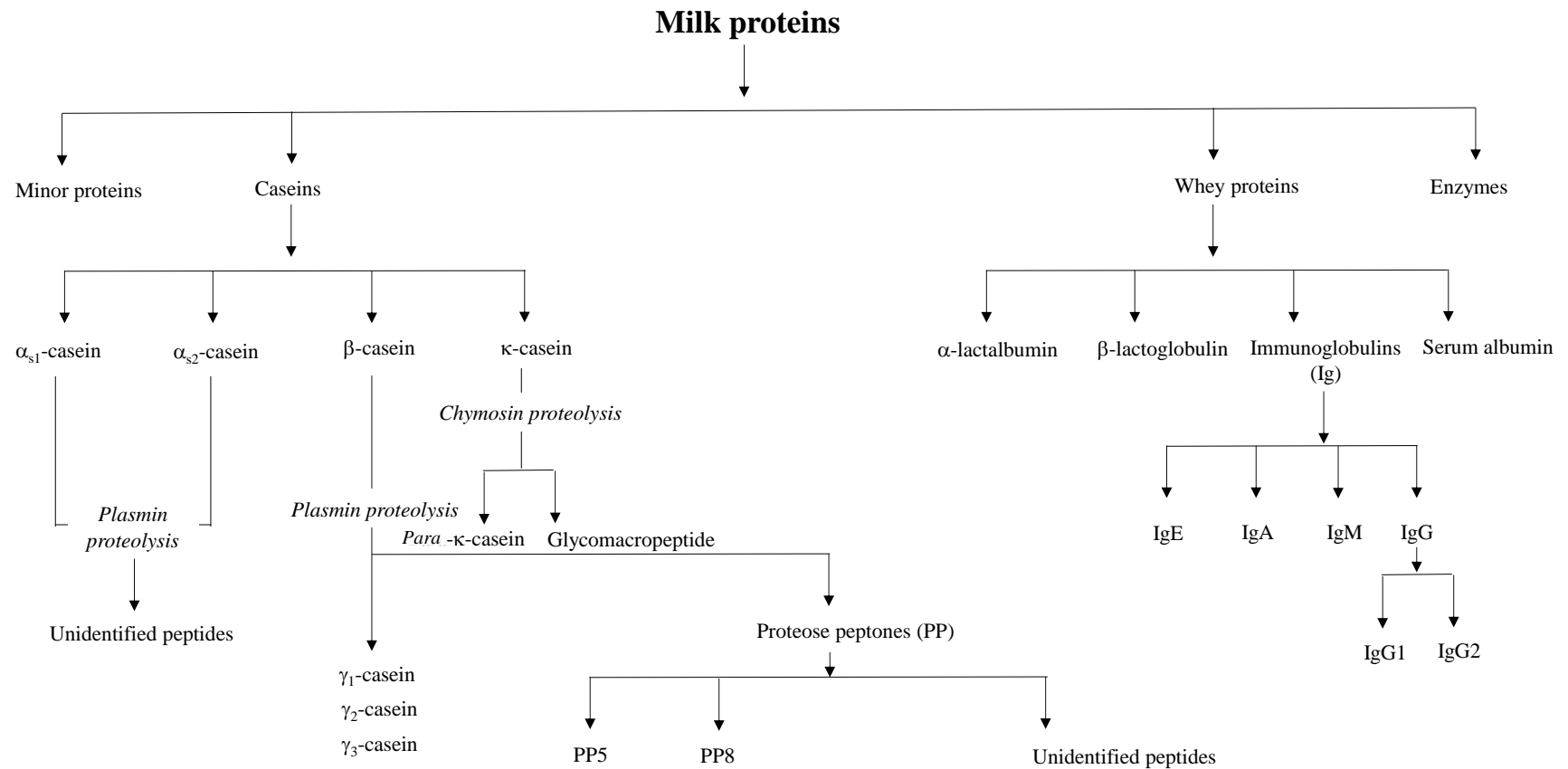
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## **1.1 Milk proteins**

Milk has a unique profile of components, including proteins, fats, lactose, minerals and other minor compounds, the primary function of which is to meet the nutritional requirements of the neonate (O'Mahony & Fox, 2013). Bovine milk contains ~2.9-3.3% protein, which is divided into two main classes; casein and whey (Table 1.1; Fig. 1.1). In mature milk, the casein fraction represents approximately ~78% of the total milk protein while the remaining ~22% is classified as whey proteins (O'Connell *et al.*, 2015; O'Mahony & Fox, 2013). In the serum phase of milk, proteins of two size ranges are present; whey proteins dissolved at the molecular level (~1-100 nm) and caseins dispersed as large (~50-500 nm) colloidal spherical aggregates (micelles), along with lipids emulsified as large (~1-20 µm) globules (Fox, 2009; Fig. 1.1). In addition to its principal components, milk contains several hundred biologically-active minor constituents, e.g., vitamins, hormones, enzymes, metal ions, flavour compounds, immunoglobulins, enzyme inhibitors, binding/carrier proteins, growth factors and antibacterial agents, which have been thoroughly reviewed by several authors, including Farrell (2002) and Coscia *et al.* (2012).

### *1.1.1 Caseins*

Isoelectric precipitation is commonly used to separate the casein and whey protein fractions in milk, as the caseins are insoluble at their isoelectric point (pH ~4.6) at temperatures >~6°C, while native whey proteins remain soluble (O'Mahony & Fox, 2013).



**Fig. 1.1.** Schematic representation of the heterogeneity of protein in bovine milk. Differences due to genetic variations, variable phosphorylation and glycosylation are not shown. Adapted from Mulvihill and O'Donovan (1987) and Swaisgood (1993).

**Table 1.1.** Typical gross composition of mature bovine milk (adapted, with minor modifications, from O' Regan et al., 2009).

Component			Concentration (g/L milk)	Molecular Weight (kDa)
Total Protein			29-33	-
Caseins	Main caseis proteins	$\alpha_{s1}$ -casein	9-15	23.6
		$\beta$ -casein	9-11	24.0
		$\kappa$ -casein	3-4	19.6
		$\alpha_{s2}$ -casein	3-4	25.2
	Proteose-peptones	Proteose-peptones	0.6-1.8	4-40
Whey	Main whey proteins	$\beta$ -lactoglobulin	2-4	18.4
		$\alpha$ -lactalbumin	0.7-1.5	14.2
		Serum albumin	0.1-0.4	66.3
	Immunoglobulins	IgG1, IgG2	0.65	~150
IgA		0.14	~385	
IgM		0.05	~900	
	Minor Proteins	Lactoferrin	0.8	~80
Transferrin		0.1	86	
	Milk fat globule membrane proteins		0.1	~35-90
	Fat		37-50	-
	Lactose		47-50	-
	Ash		6.8-7.4	-
	Water		845-877	-

Caseins are synthesised exclusively in the mammary gland and are a heterogeneous group of proteins comprised of four main fractions, i.e.,  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -casein, which are present in the relative proportions 37:10:35:12 (Holt & Sawyer, 1993). However, polyacrylamide gel electrophoresis of caseins has

indicated much greater heterogeneity, which is due to relatively small variations in one or more of the four principal caseins (Heck *et al.*, 2008; Swaisgood, 1993). Milk contains several indigenous proteinases, the most important of which is plasmin, a trypsin-like, serine-type proteinase from blood with a high specificity for peptide bonds containing lysine or arginine (Fox & Kelly, 2006; O'Mahony *et al.* 2013). All the casein protein fractions contain several lysine and arginine residues, but only a few bonds are hydrolysed rapidly. Hydrolysis of  $\beta$ -casein by plasmin can result in the liberation of C-terminal peptides which are known as  $\gamma$ -caseins ( $\gamma_1$ :  $\beta$ -casein f29–209;  $\gamma_2$ :  $\beta$ -casein f106–209;  $\gamma_3$ :  $\beta$ -casein f108–209; Kelly & McSweeney, 2003), and proteose-peptones (Fig. 1.1) (Andrews, 1978).  $\kappa$ -Casein is sensitive to hydrolysis by chymosin at the Phe<sub>105</sub>-Met<sub>106</sub> bond resulting in two hydrolysis products being released; (1) hydrophobic *para*- $\kappa$ -casein (resides 1-105), which consists of two cysteine residues, and (2) hydrophilic glycomacropeptide (GMP) that contains all of the phosphate and glycosyl groups and no aromatic amino acids.

### *1.1.2 Casein Micelle*

In milk, ~95% of total casein is typically arranged as colloidal, roughly spherical aggregates, known as casein micelles, which, molecularly, are relatively large ( $10^5$  - $10^6$  kDa) and contain, on a dry matter basis, ~94% protein and ~6% colloidal calcium phosphate. One of the main biological functions of caseins is to act as sequestrants of colloidal calcium phosphate for nutrition of the neonate (Holt *et al.*, 1996, 1998). Various models of casein micelle structure have been proposed and reviewed by several authors over the last ~50 years (Dalglish, 2011; Fox & Brodtkorb, 2008; Horne, 1998; McMahon & Brown, 1984; Morr, 1967; O'Mahony & Fox, 2013). It is generally accepted that  $\kappa$ -casein (representing ~12-15% of total

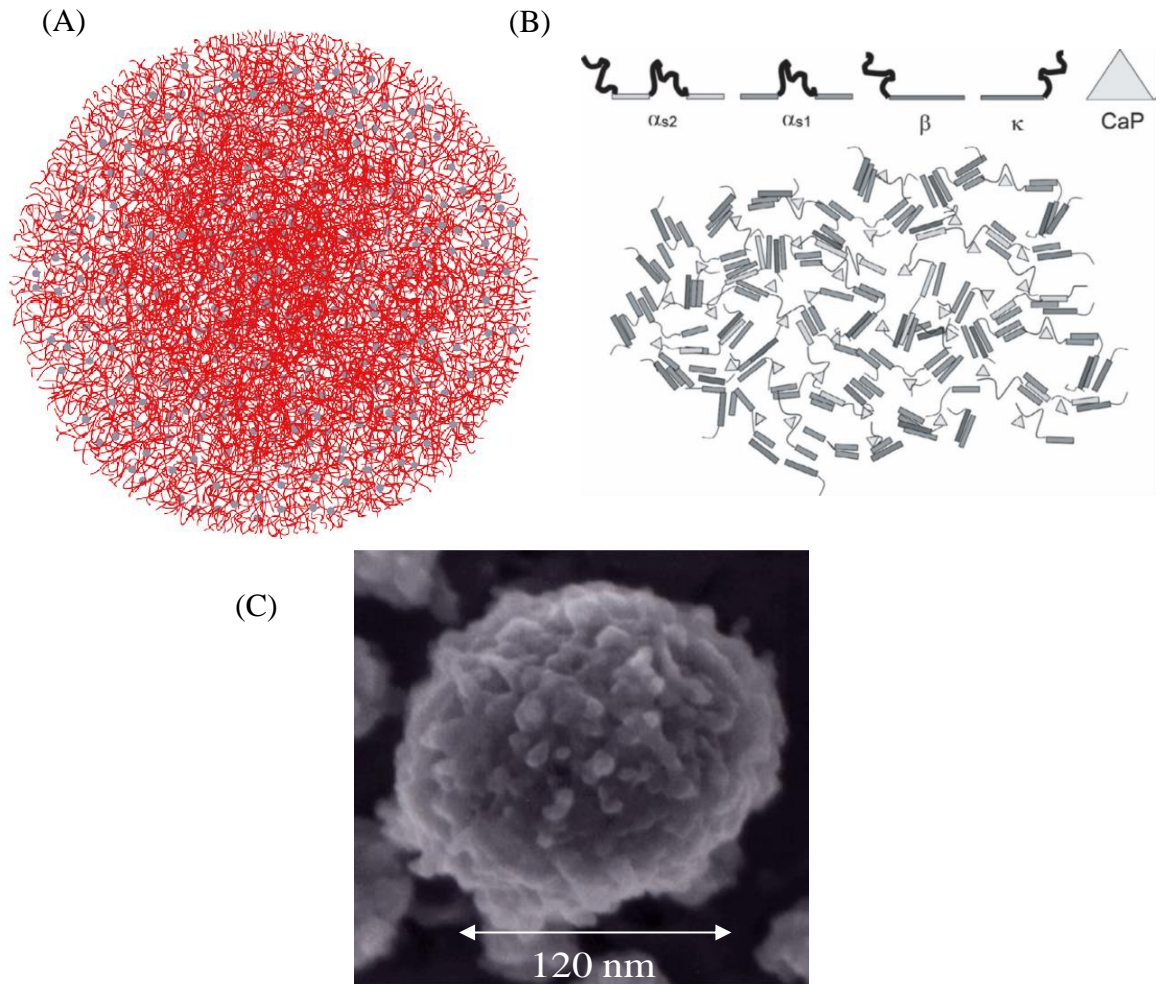
casein) is preferentially located at the surface of the micelle so that it can stabilise the calcium-sensitive  $\alpha_{s1}$ -,  $\alpha_{s2}$ - and  $\beta$ -caseins (representing ~85% of total casein) *via* electrostatic and steric repulsion (Crowley *et al.*, 2016).

Fig. 1.2 shows a schematic representation of the hairy casein micelle model proposed by Holt (1992), who depicted the casein micelle as a tangled web of flexible casein molecules forming a gel-like structure in which micro-granules of colloidal calcium phosphate are an integral feature, from the surface of which the C-terminal region of  $\kappa$ -casein extends, forming a “hairy layer”, 12 +/- 2 nm thick (Holt & Dalgleish, 1986). A ‘dual-bonding’ model of casein micelle structure has been proposed and developed by Horne (1998) and suggests that micelle structure is governed by a balance between hydrophobic and electrostatic interactions and colloidal calcium phosphate-mediated cross-linking of hydrophilic regions, which contain seryl phosphate residues (Fig. 1.2). All four caseins have phosphoseryl residues, with the phosphoseryl residues in  $\alpha_{s1}$ -,  $\alpha_{s2}$ - and  $\beta$ -caseins occurring as clusters; these caseins have 2, 3 and 1 cluster(s) of phosphoryl residues, respectively, and these caseins form a network by intermolecular hydrophobic interactions and colloidal calcium phosphate crosslinks.  $\kappa$ -Casein which has only 1 phosphoseryl residue, has no such cluster and functions as a polymerization blocker.

### *1.1.3 Techno-functional properties of caseins*

Caseins are very flexible molecules, which is attributed to the high content of the structure-disrupting amino acid, proline, present at high levels in  $\beta$ -casein with both  $\alpha_{s1}$ - and  $\beta$ -casein lacking cysteine (Table 1.2). The open and flexible conformation of casein molecules, which has been termed ‘rheomorphic’, is environment-

dependent and has been thoroughly described by several authors (Holt & Sawyer, 1993; Horne, 2002; Velev, 1998; Walstra, 1990).



**Fig. 1.2.** (A) Schematic representation of the hairy casein micelle model proposed by Holt; polypeptide chains (red threads), colloidal calcium phosphate (grey circles) (from Holt, 2016). (B) The dual binding model as proposed by Horne; hydrophobic regions (rectangular bars) (from Horne, 1998). (C) High-resolution field-emission scanning electron micrograph of a single casein micelle (from Dalgleish et al., 2004).

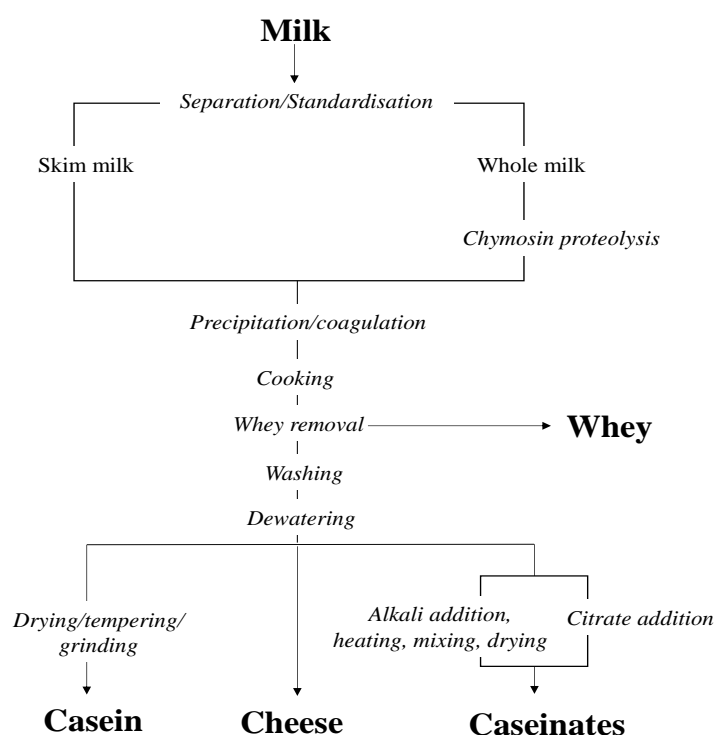


Caseins are generally referred to as hydrophobic proteins; however, with the exception of  $\beta$ -casein, they do not contain a high proportion of hydrophobic amino acids; instead caseins have a high surface hydrophobicity due to their lack of stable secondary and tertiary structures (Crowley *et al.*, 2016). The low level of secondary structure of caseins also contributes to their good heat stability; milk at pH 6.7 can be heated at 140°C for up to ~25 min without coagulation. The hydrophobic and hydrophilic regions in caseins are not distributed evenly, giving the caseins a distinctly amphipathic structure (Huppertz, 2016). The amphipathic structure feature, in combination with their rheomorphic conformation, provides the caseins with excellent foaming, emulsifying, heat stability and gel-forming properties but also renders them susceptible to proteolysis, an attribute that is exploited during the manufacture of most cheeses and rennet casein. Caseins can be separated from bovine milk, following coagulation of the casein proteins through the action of chymosin, mineral or acid addition or directly by using microfiltration membranes with the remaining yellow/green watery liquid known as whey (Smithers, 2008, 2015).

In the cheese-making process, casein is destabilised by the action of chymosin, with a coagulum formed consisting of casein, some whey proteins, fat and the minerals of the milk (Fig. 1.3). Casein-based ingredients can be divided into the following types of products; (1) rennet casein, obtained by enzymatic precipitation by chymosin (rennet) addition where the enzyme specifically cleaves Phe<sub>105</sub>-Met<sub>106</sub> bond in  $\kappa$ -casein (Section 1.1.1) which destabilises the casein micelles and forms a three-dimensional coagulum with calcium ions and (2) acid casein, produced by acidifying skim milk (e.g., lactic acid, HCl, H<sub>2</sub>SO<sub>4</sub>) to the isoelectric point (pH 4.6 – 4.7) resulting in a casein precipitate (O'Regan & Mulvihill, 2009). Acid casein is

insoluble in water and is generally considered a precursor ingredient for the manufacture of caseinates; it may be converted to water-soluble caseinates by dispersion in water and adjusting the pH to ~ 6.7 with alkali, usually with NaOH to yield sodium caseinate.

KOH,  $\text{NH}_4\text{OH}$  or  $\text{Ca}(\text{OH})_2$  may also be used, giving the corresponding caseinate (Mulvihill & Ennis, 2003). The heat stability of sodium caseinate is relatively high, but can be markedly reduced in the presence of ionic calcium. The addition of calcium-chelating salts, such as citrates, can improve the heat stability and the solubility of caseinates by modifying the mineral equilibrium (O'Regan & Mulvihill, 2009). In addition to these main types of casein and caseinates, there are



**Fig. 1.3.** Simplified overview of casein-based products obtained from milk (from O'Regan and Mulvihill, 2009).

other commercially available casein products of importance, such as co-precipitates, made by heating skim milk to a high temperature and then precipitating the casein/whey protein complex, usually with calcium chloride (O'Regan & Mulvihill, 2009).

Milk protein concentrates and isolates (MPCs and MPIs) are produced by heat-treating skim milk and concentrating both whey protein and casein fractions in the retentate using ultrafiltration (UF) and sometimes diafiltration (DF) (Mulvihill & Ennis, 2003). MPCs are classified according to their protein content, with MPC50 being an example of a low-protein MPC (~50% protein) and MPC80 representing a high-protein MPC (~80% protein). The use of a membrane process largely maintains the two major protein families in their native states (i.e., undenatured structure, low levels of process-induced covalently-bonded aggregates) and at their natural ratio (78:22 casein:whey protein). MPCs have a more favourable sensory profile compared to other casein-dominant ingredients, such as caseinates, acid casein and rennet casein (Smith *et al.*, 2016). High-protein MPCs/MPIs are a key component of several nutritional beverages, including meal-replacers to prevent malnutrition in clinical environments, therapeutic drinks for the control of ageing-related diseases such as sarcopenia and lactose-free infant milk formulae. Phosphocaseinates, also termed native phosphocaseinates, native micellar caseins or micellar casein concentrates, are comprised primarily of the casein fraction, and associated minerals, retained following microfiltration (MF) or a combined MF/DF process (Schuck, 2013).

#### *1.1.4 Liquid whey*

Whey removed after starter culture and chymosin addition (i.e., the co-product of rennet coagulated cheese and rennet casein manufacture, respectively) is referred to as ‘sweet whey’ and is the principal raw material from which whey protein ingredients are manufactured. Although sweet whey is a commonly used starting material for the production of whey protein ingredients, the presence of colours (e.g., annatto) or bleaching residues (e.g., benzoyl peroxide or hydrogen peroxide) may be of concern for infant formula manufacturers (Crowley *et al.*, 2016). The functional properties of whey protein ingredients produced from sweet whey containing glycomacropeptide (GMP) are different to those of whey without GMP due to differences in heat-induced denaturation–aggregation profiles as detailed in Section 1.4 (Outinen & Rantamäki, 2008; Croguennec *et al.*, 2014). Furthermore, self-association of GMP has also been implicated in the development of turbidity during the storage of acidic whey protein beverages (Villumsen *et al.*, 2015).

Acid whey is a by-product of the manufacture of products involving fermentation of lactose to lactic acid or by direct addition of organic (e.g., citric, lactic) or mineral (e.g., sulphuric, phosphoric, hydrochloric) acids to a pH of ~4.6–5.0 (Bansal & Bhandari, 2016). Acid-induced coagulation significantly increases the amount of calcium in whey due to changes in the equilibrium of the milk salts system. At low pH, colloidal calcium phosphate in casein micelles shifts equilibrium towards solubilised calcium and phosphorus, with these ions being present in the whey during separation, leading to the high calcium and phosphate content of acid whey (Wong *et al.*, 1978). Typically, acid whey is produced from fresh cheese and Greek-style yoghurt manufacture (e.g., Cottage cheese, stirred yoghurt) or from direct acidification by organic (e.g., lactic) or mineral (e.g., HCl) acid during casein

production. Acid whey from Greek yoghurt production is often not ideal for the production of protein ingredients, as it contains little protein (<0.1%) and has high levels of minerals, lactic acid and galactose, which can cause processing issues, particularly during spray drying (Elliott, 2013; Lucey, 2016).

## **1.2 Principal Whey Proteins**

### *1.2.1 Whey proteins*

Bovine whey protein consists primarily of  $\beta$ -lactoglobulin (~50%),  $\alpha$ -lactalbumin (~20%), blood serum albumin (~1%), immunoglobulins (~3%), lactoferrin (~1%) proteose-peptone fractions (~10%), and GMP (~15-20%, present only in sweet whey) (Farrell *et al.*, 2004; O'Mahony & Fox, 2013), along with more than 200 different minor whey proteins (e.g.,  $\beta_2$ -microglobulin, osteopontin, kininogen), the majority of which are present in trace amounts (Golinelli *et al.*, 2011). Whey proteins, in their native form, are compact globular structures with non-polar groups, such as hydrophobic amino acids, mostly arranged in the centre of folded peptide chains. Interestingly, from the point-of-view of conjugation, the whey proteins generally have slightly higher normalised levels of lysine residues than the caseins (Table 1.2).

### *1.2.2 $\beta$ -Lactoglobulin*

Bovine  $\beta$ -lactoglobulin ( $\beta$ -lg) belongs to the lipocalin family, a widely-studied group of proteins characterised by their ability to transport hydrophobic ligands (Sawyer, 2013) and some of the key physicochemical properties of this protein are presented in Table 1.2. Le Maux (2014) summarised the physiological functions of  $\beta$ -lg

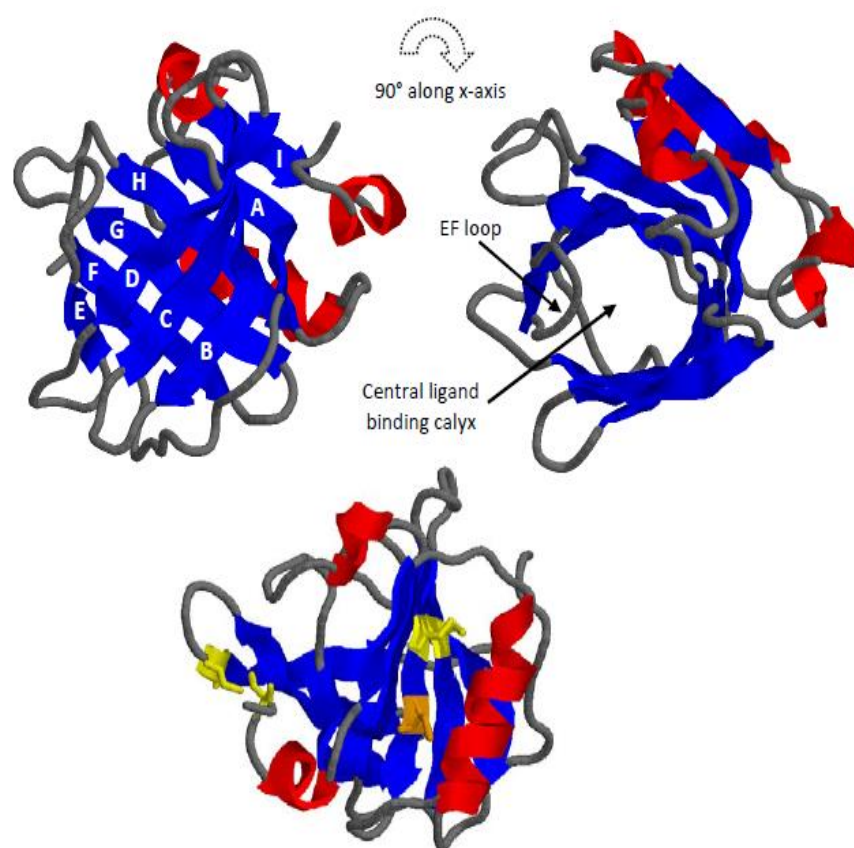
including, (1) increasing fatty acid absorption in the intestine, (2) protection of sensitive ligands (e.g., retinol) against oxidation or other stresses, (3) modification of the bio-accessibility of ligands and (4) nutritional contribution of the individual components (i.e.,  $\beta$ -lg and ligands).  $\beta$ -Lg exists mainly as two genetic variants (A and B) differing from each other by the substitution of 2 amino acids; the aspartic acid residue at position 64 and valine residue at position 118 in genetic variant A are substituted by glycine and alanine, respectively, in genetic variant B.

$\beta$ -Lg is a highly structured protein; in the pH range 2–6, the secondary structure of the molecule exists as approximately 10–15%  $\alpha$ -helix, 43%  $\beta$ -sheet, which occurs in a  $\beta$ -barrel-type calyx, and 47% unordered structures. The tertiary structure of  $\beta$ -lg is very compact and globular, in which eight out of nine anti-parallel  $\beta$ -sheets (Fig 1.2, A-I) wrap around to create a flattened, conical  $\beta$ -calyx which can bind ligands (Papiz *et al.*, 1986).

There is a loop at the E- and F-strands (EF-loop), the configuration of which is pH-dependent and effectively acts as a lip, opening and closing the central ligand-binding calyx (Fig. 1.4) (Ragona, 2003). The quaternary structure of  $\beta$ -lg varies with pH; at pH <3.5 and pH >7.5,  $\beta$ -lg exists as a monomer with a molecular weight ( $M_w$ ) of 18.4 kDa; however, in the pH range 5.5–7.5,  $\beta$ -lg exists as a dimer of  $M_w \sim 36$  kDa.  $\beta$ -lg also contains five cysteine (Cys) residues, which form two intramolecular disulphide bonds along with a free thiol group (Cys<sub>121</sub>) that lies buried in the centre of the native  $\beta$ -lg structure.

Under native conditions, the free thiol group at Cys<sub>121</sub> is unavailable for reaction as it is buried in the centre of the molecule but under conditions that induce protein unfolding (i.e., > 70°C, extremes of pH or high pressure), the tertiary

structure of  $\beta$ -lg can partly unfold, exposing the free thiol group, making it available for reaction with other thiol groups *via* disulphide bridging or intra/inter-molecular disulphide interchange (Croguennec *et al.*, 2004; Papiz *et al.*, 1986).



**Fig. 1.4.** Structure of  $\beta$ -lactoglobulin;  $\alpha$ -helix are shown in red,  $\beta$ -sheet are shown in blue, the four cysteine residues involved in disulphide bonding are shown in yellow and the reactive thiol group (Cys 121) is shown in orange (from O'Loughlin *et al.*, 2014).

**Table 1.2.** Selected composition and physicochemical characteristics of casein and whey protein molecules. Adapted with modifications from Huppertz (2016) and O'Regan, Ennis and Mulvihill (2009).

<i>Component</i>	<i>Number of amino acids</i>	<i>Isoelectric pH*</i>	<i>Number of Proline residues</i>	<i>Number of Lysine residues</i>	<i>Normalised number of lysine residues (per 100 residues)</i>	<i>Number of Cysteine residues</i>
$\alpha_{s1}$ -casein	199	4.4-4.8	17	14	7.0	0
$\alpha_{s2}$ -casein	207	-	10	24	11.6	2
$\beta$ -casein	209	4.8-5.1	35	11	5.3	0
$\kappa$ -casein	169	5.3-5.8	20	9	5.3	2
$\beta$ -lactoglobulin	162	5.2-5.8	8	16	9.9	5
$\alpha$ -lactalbumin	123	4.3-4.7	2	13	10.6	8
Blood serum albumin	582	4.7-4.9	28	60	10.3	35

*\*genetic variations and variable phosphorylation account for some of the ranges given*



### 1.2.3 $\alpha$ -Lactalbumin

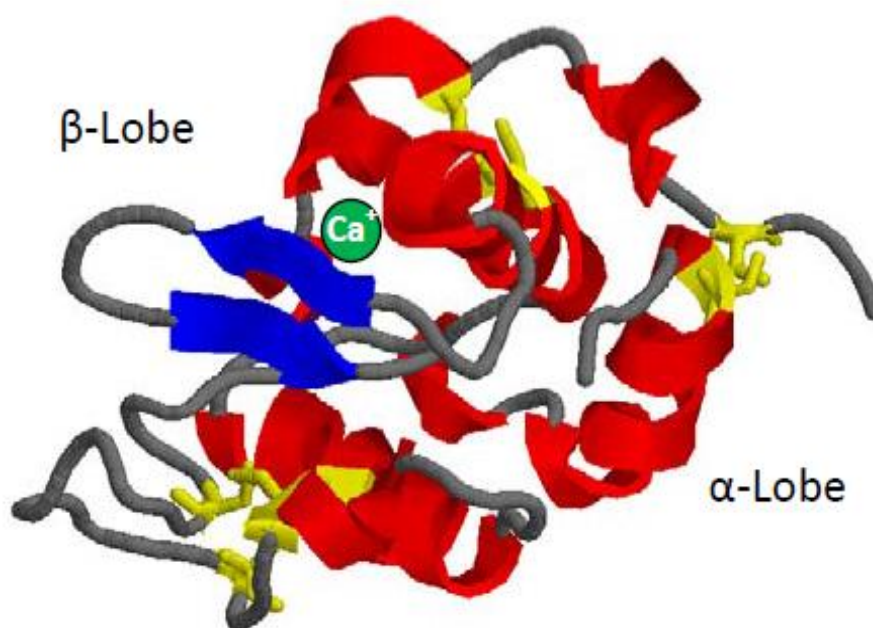
$\alpha$ -Lactalbumin ( $\alpha$ -lac) comprises ~15-20% of total whey protein and is a small, highly structured, globular protein (Brew, 2013; Table 1.2). It contains four tryptophan residues per molecule, which play an important role in the synthesis of serotonin, a potent neurotransmitter. The biological function of  $\alpha$ -lac is to modify the activity of lactose synthase, an enzyme which catalyses the final step in the biosynthesis of lactose (Bleck *et al.*, 2009).  $\alpha$ -Lac is a metalloprotein; it can exist in a calcium bound (holo- $\alpha$ -lac) form, which contains 2 calcium ions ( $\text{Ca}^{2+}$ ) per molecule in a pocket containing 4 aspartate residues, or a calcium-depleted form (apo- $\alpha$ -lac), as when the pH is reduced to <5, the aspartate residues become protonated and lose their ability to bind calcium.

The holo- $\alpha$ -lac form is the most heat-stable with a denaturation temperature of 62°C and the protein has the ability to renature following heat denaturation. The apo- $\alpha$ -lac form has been reported to denature at temperatures as low as 35°C, depending on the ionic environment and pH and does not renature on cooling due to the strong repulsive charges that exist between the carboxyl groups of the aspartate residues in the absence of  $\text{Ca}^{2+}$  (Hendrix *et al.*, 2000). These calcium-dependant heat- and pH-sensitivities of  $\alpha$ -lac have been exploited to enrich/isolate  $\alpha$ -lac from liquid whey;  $\alpha$ -lac is precipitated by acidification of whey using organic acids, citric and lactic acids at 50°C, pH 4, and with control of calcium concentration at an organic acid/ $\text{Ca}^{2+}$  molar ratio higher than 9 (Kamau, 2010; Pearce, 1983).

Nieuwenhuizen *et al.* (2003) reported that the availability of the lysine groups in  $\alpha$ -lac are modified by the binding of calcium; five lysine residues were available for

reaction in apo- $\alpha$ -lac compared to four available lysine residues in the halo- $\alpha$ -lac.  $\alpha$ -Lac contains 8 cysteine residues which form 4 intramolecular disulphide bonds that confer structural rigidity to the compact globular structure of the protein (Pike *et al.*, 1996). However, there are no Cys residues with free sulphhydryl groups in  $\alpha$ -lac, which increases its thermal stability relative to  $\beta$ -lg due to the increased ability of holo- $\alpha$ -lac to renature following heat treatment relative to  $\beta$ -lg.

The secondary structure of the protein consists of 29%  $\alpha$ -helix, 18%  $\beta$ -sheet and 53% non-repeating sequence (Brew, 2013). The globular tertiary structure of the  $\alpha$ -lac molecule is divided into two lobes; one of these domains is rich in  $\alpha$ -helix (the  $\alpha$ -lobe) and has disulphide bonds at Cys<sub>6</sub>-Cys<sub>120</sub> and Cys<sub>28</sub>-Cys<sub>111</sub>. The other domain is rich in  $\beta$ -sheet (the  $\beta$ -lobe) and has disulphide bonds at residues Cys<sub>61</sub>-Cys<sub>77</sub> and Cys<sub>73</sub>-Cys<sub>91</sub>, and includes one calcium binding site (Hendrix, 1996; Pike *et al.*, 1996). The cleft that separates the lobes in  $\alpha$ -lac is not open at both ends, and the Ca<sup>2+</sup> binding elbow is located at the junction of the subdomains (Fig. 1.5).  $\alpha$ -Lac has also demonstrated binding affinities for other polyvalent cations including Mg<sup>2+</sup>, Mn<sup>2+</sup> and Zn<sup>2+</sup>. Interestingly, in recent years, it has been reported that bovine  $\alpha$ -lac can be converted to a form that has anti-carcinogenic activity known as BAMLET (bovine  $\alpha$ -lac made lethal to tumour cells) (Lišková *et al.*, 2010).



**Fig. 1.5.** Structure of  $\alpha$ -lactalbumin;  $\alpha$ -helix is shown in red,  $\beta$ -sheet is shown in blue, the eight cysteine residues and side chains are shown in yellow (from O'Loughlin *et al.*, 2014).

### 1.2.3 Bovine serum albumin

Bovine serum albumin (BSA) has 582 amino acids and a  $M_w$  of 66.3 kDa (Table 1.2). Unlike  $\alpha$ -lac and  $\beta$ -lg, BSA is not synthesised in the mammary gland, but is present in milk due to passive leakage from blood. BSA contains high levels of lysine, leucine, alanine and glutamate and possess three structural domains that bind lipids, metals and nucleotides, respectively. BSA is a monomeric protein with 17 disulphide bonds and 1 free Cys. Its denaturation temperature is  $\sim 60^\circ\text{C}$ , although its thermo-stability can be increased after binding some hydrophobic ligands (Hayakawa *et al.*, 1992).

### 1.2.5 Immunoglobulins

The immunoglobulins (Ig), of which there are three major classes in milk, i.e., IgG, IgM and IgA, account for about 1% of the total milk protein or about 6% of the total whey protein (Farrell *et al.*, 2004). Factors which affect the Ig concentrations in milk include breed, age of the cow, lactation number and volume of the first *post-partum* milking and their primary function is to supply passive immunity to the neonate *via* maternal antibodies. IgG1 is the principal Ig in bovine milk, with lesser amounts of IgG2, IgA and IgM. Structurally, IgGs consist of 2 heavy chains (~60 kDa) and 2 light chains (~22 kDa) linked by a number of disulphide bonds and are the most thermally stable of the immunoglobulins.

### 1.2.6 Glycomacropeptide

GMP is a hydrophilic, unfolded, peptide, which is released when  $\kappa$ -casein in milk is hydrolysed with chymosin during cheese and rennet casein production, producing *para*- $\kappa$ -casein and a smaller peptide from the C-terminal region of  $\kappa$ -casein (i.e., GMP). Several authors have used the term “caseinomacropeptide” interchangeably with GMP; however in this review the latter term will be used exclusively to refer to the hydrophilic peptide cleaved from  $\kappa$ -casein. GMP has a monomeric  $M_w$  of ~8 kDa; however, due to genetic variability and post-translational modifications (e.g., glycosylation, phosphorylation), the  $M_w$  can range from ~25-30 kDa. GMP comprises 15-20% of the total protein in cheese whey and contains no aromatic residues (e.g., Phe, Try and Tyr), only one sulphur-containing amino acid (Met) and three lysine (Lys) residues in its sequence but high levels of branched chain amino acids (e.g., Ile and Val) (Tolkach & Kulozik, 2005). The isoelectric point of

GMP varies from 4.15 (non-glycosylated) to 3.15 (glycosylated) due to the presence of sialic acid in the glycan moieties (Kreuß *et al.*, 2009).

#### 1.2.7 Proteose-peptone

Proteose-peptone (PP) refers to the fraction of milk protein chemically defined as a complex mixture of phosphoproteins, glycoproteins and peptides, which remain soluble at pH 4.6, following heat treatment of whey at 95-100°C for 30 min (Mulvihill & O'Donovan, 1987; Rowland, 1938). PP can also be precipitated by 12% trichloroacetic acid. The PP fraction of bovine milk represents about 10% of the pH 4.6-soluble nitrogen of whey protein and is quite heterogeneous but can be characterised into two broad groups; (1) those derived by proteolysis of caseins and (2) minor indigenous milk proteins such as osteopontin and PP3, also known as lactophorin (Fox, 2009).

#### 1.2.7 Other whey protein fractions

In addition to its principal proteins, milk contains several hundred minor protein-based constituents, including lactoferrin, lactoperoxidase, lysozyme and other minor whey proteins and growth factors which have been comprehensively reviewed by several authors including Crowley *et al.* (2016), Hurley (2003), Peters (1985), Tomita *et al.* (2002) and Wynn and Sheehy (2013). Although these proteins have important biological functions and some of their key properties are outlined in Table 1.1 and 1.2, their low concentration in milk means that they have little effect on the physicochemical and technological properties of whey protein-based ingredients such as whey protein concentrate (WPC) and whey protein isolate (WPI).

### 1.3 Commercial whey protein ingredients

The proteins of milk are considered its most valued constituents from both a techno-functional and nutritional perspective and many whey protein-based ingredients have been developed over the years that harness the unique functional and nutritional properties of whey proteins (Fig. 1.6). In essence, the production of whey protein ingredients involves the reduction of the water content of liquid whey and other components (e.g., lactose, minerals and organic acids) to increase the relative levels of the whey proteins (Morr & Ha, 1993). Variations in the composition and functionality of whey protein ingredients are typically attributed to differences in the liquid whey composition as well as the processing conditions used in their manufacture (Schmidt *et al.*, 1984). The production of whey protein ingredients relies on the use of cost-effective and commercially-scalable processes to yield standardized products with consistent composition and quality (Smithers, 2015).

The development of pressure-driven cross-flow membrane filtration technology has facilitated the production of a variety of highly functional whey protein ingredients. Membrane technology can be used for pre-concentration of whey proteins prior to evaporation/spray drying along with removal of ions and purification/enrichment of proteins and peptides. Selection of the appropriate membrane  $M_w$ /pore size cut-off, along with varying the environmental conditions (e.g., temperature, ionic strength and pH) of the solution during processing can also enrich specific protein fractions (Daufin *et al.*, 2001). Membrane filtration including MF, UF, nanofiltration (NF) and reverse osmosis (Fig. 1.7) may be combined with DF to achieve higher protein concentrations (Gésan-Guiziou, 2013; Mulvihill & Ennis, 2003).



A whey stream extracted directly from milk using microfiltration (MF) membranes to concentrate and separate whey proteins in their native form is known as 'ideal' or 'native' whey. This production of ideal whey is of growing interest due to its unique properties, which arise from the milk having not been subjected to excessive heating, the action of rennet, starter cultures, colours or acid. As a result of this, there is an absence of GMP, less lactic acid, no degradation of proteins by starter culture enzymes and the risks from bacteriophages are prevented along with the greater retention of native whey protein conformation (Daufin *et al.*, 2001; Heino *et al.*, 2007).

### 1.3.2 Whey powder

Liquid whey (~6.5% total solids) generally contains casein curd fines and whey cream that need to be removed by centrifugation and separation to minimize the risk of blockage of the heat exchangers or damaging filtration membranes during further processing (Kjaergaard *et al.*, 1988; Jelen, 2002). Sweet whey is pasteurized (72-75°C for 15-20 s) for optimum microbiological quality and storage stability and is then typically cooled to 60-65°C and held at temperature for 30-60 min to stabilise the calcium-phosphate complex which minimises fouling during further membrane processing steps (Schuck, 2013).

Liquid sweet whey can also be pre-treated to concentrate (i.e., remove water) by reverse osmosis (RO) (Bansal & Bhandari, 2016). Following this, the liquid whey is concentrated by evaporation, cooled, seeded with lactose crystals and held with agitation to promote crystallisation of lactose before being dried (Jelen, 2009). Standard whey powder can be classified according to the source of liquid whey (i.e.,

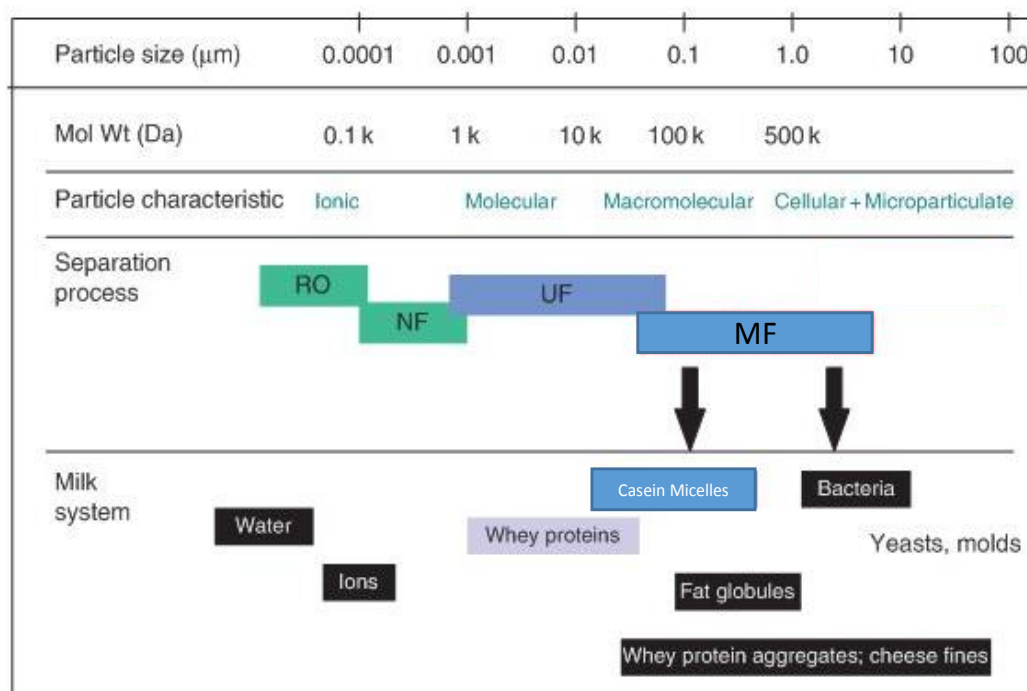


sweet/acid whey powder) (Bhandari *et al.*, 2008). High levels of lactic acid (>2%) may result in processing issues, particularly during spray drying, as lactic acid is thermoplastic and can lower the glass transition temperature of lactose (Bylund, 2003). Due to the high levels of lactose (70-80%), whey powders are commonly used in bakery and confectionary products as they readily undergo Maillard browning. The typical composition of whey protein ingredients with protein content of 12-90% are shown in Table 1.3.

### *1.3.3 Demineralised whey*

For certain applications (e.g., infant milk formula), it may be desirable to reduce the innate mineral concentrations of a whey protein ingredient for physiological, functional or sensory reasons (Jost *et al.*, 1999). Partial (<70%) or extensive (~90%) demineralisation of liquid whey can be completed by using mixed bed cation/anion exchange chromatographic resins, electrodialysis (ED), NF or a combination of these unit operations (Bansal & Bhandari, 2016).

ED typically removes monovalent ions (e.g.,  $K^+$ ,  $Na^+$ ,  $Cl^-$ ) while NF can remove monovalent ions along with the simultaneous removal of water and ion exchange chromatography (IEX) has the ability to selectively remove all the minerals (e.g., mono/di/polyvalent ions) (Atra *et al.*, 2005). By controlling the extent of ion removal, whey powders with varying degrees of demineralisation can be produced which are typically spray dried to powder format (Ayala-Bribiesca *et al.*, 2006).



**Fig. 1.7.** Overview of filtration processes including reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF) and microfiltration (MF) applied in the dairy industry showing the relative molecular weight sizes of typical milk constituents (from Pouliot, 2008).

#### 1.3.4 Whey protein concentrate (WPC)

Several protein ingredients including high-protein products such as whey protein concentrates (WPC) can be generated from pre-treated liquid whey using membrane filtration. These WPC products are produced by UF of liquid whey using a membrane with  $M_w$  cut-off of 5-20 kDa to separate low  $M_w$  components (i.e., lactose, salts and water) in the permeate from whey protein molecules which are large enough to be retained (Brans *et al.*, 2004).

Using a single-stage, a typical UF process is able to concentrate whey proteins to 10-30 times the concentration in the feed. However, in combination with diafiltration (DF), a process in which water is added to the feed as filtration proceeds

to wash out additional low  $M_w$  components, large volumes of liquid whey can be processed by UF membranes allowing for the concentration of whey protein components to 35-75% on a dry matter basis. The desired protein concentration is achieved by controlling the membrane pore size, the volume concentration factor and the use of DF (Pouliot, 2008). Further concentration and removal of water is completed by evaporation and spray drying.

The application of instantisation-enabling technologies (i.e., agglomeration, lecithination) to high protein whey ingredients (e.g., WPC80, WPC85) is commonly practiced, prompted by the dramatic growth in demand for fast-dissolving protein ingredients for use in sports nutrition applications (Wright *et al.*, 2009). Recent advances in membrane materials and plant construction and operation has allowed for UF/DF of whey under cold conditions ( $<10^{\circ}\text{C}$ ) that limits whey protein denaturation and favours retention of functional properties (Daufin *et al.*, 2001; ATRA *et al.*, 2005; Govindasamy-Lucey *et al.*, 2007).

#### 1.3.5 Whey protein isolate

Whey protein isolate (WPI) is a protein enriched ingredient ( $>90\%$  protein) which can be prepared by (1) a combination of UF, DF and MF or (2) ion exchange chromatography. The use of UF, DF and MF for the production of WPI requires a higher UF volume concentration factor, the removal of fat (which is co-concentrated with protein) by MF and more extensive DF compared to the process used for WPC manufacture (Brans *et al.*, 2004; Kosikowski, 1979). During IEX, whey proteins are adsorbed onto anion/cation exchangers as the proteins are charged at a pH greater than or lower than their isoelectric point.

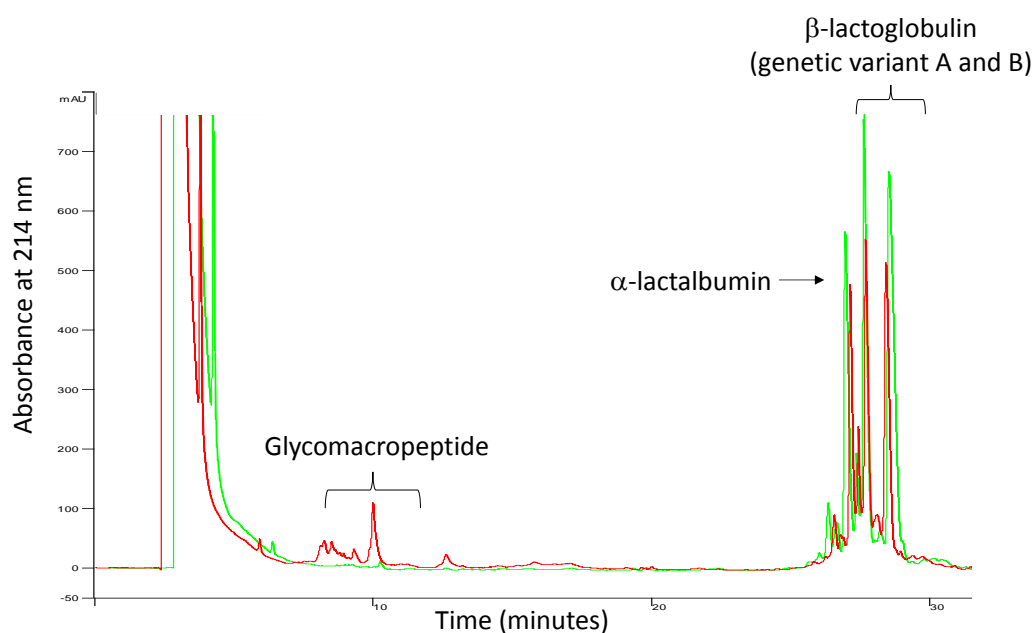
**Table. 1.3.** Typical gross composition of different whey powder products (from Bansal & Bhandari, 2016)

Component	Sweet Whey	Demineralised Whey (90%)	Whey protein concentrate	Whey protein concentrate	Whey protein isolate
	(%)				
Protein	12.0	12.0	35	80.0	90.0
Fat	<1.5	< 1.5	<5.0	<5.0	< 1.5
Lactose	83.0	74.0	50	<6.0	<2.0
Moisture	3.0	3.0	3.0	3.0	3.0
Ash	8.5	<1.0	<6.5	<4.5	<4.5

Whey protein ingredients manufactured by IEX (e.g., BiPro<sup>®</sup>, Davisco Foods International, Le Sueur, MN, USA) typically have less GMP compared to whey protein ingredients produced by membrane filtration (e.g., Carbelac<sup>®</sup>, Carbery Food Ingredients, Ballineen, Cork, Ireland) (Fig. 1.8). IEX is based on isolation of whey proteins using charge affinity and offers more flexibility to tailor the protein profile for end-use applications compared to WPI produced by membrane filtration technology that separates non-protein components and concentrates whey proteins based on molecular weight and permeation properties (Foegeding *et al.*, 2002).

### 1.3.6 Whey protein hydrolysates

Enzymatic hydrolysis of whey proteins involves the cleavage of peptide bonds resulting in the breakdown of proteins into peptides of different sizes and/or the liberation of free amino acids (Fig. 1.9). Protein hydrolysates are generally characterised by their degree of hydrolysis (DH), which expresses the number of



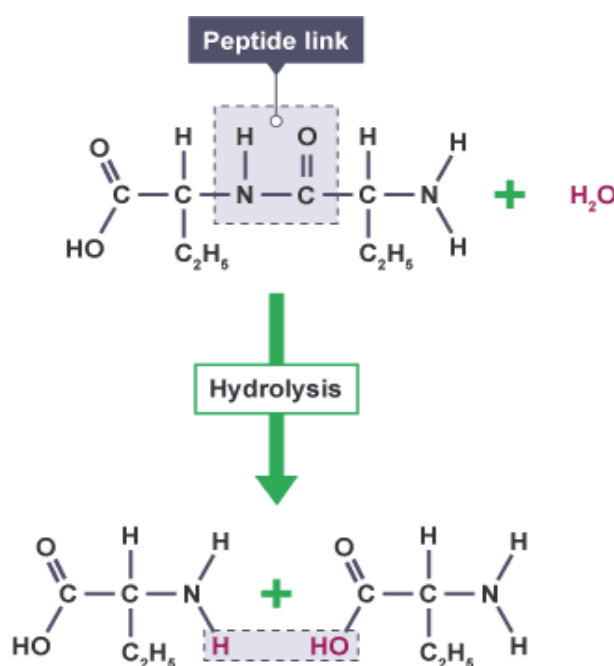
**Fig. 1.8.** Reversed-phase chromatography profile of whey protein isolate ingredients produced by with ion exchange chromatography (green) or membrane filtration (red) (unpublished data from PhD candidate).

peptide bonds cleaved as a percentage of the total number of peptide bonds available (Foegeding *et al.*, 2002). Whey protein hydrolysates (WPH) can be produced by batch or continuous hydrolysis using proteolytic enzymes (e.g., trypsin, chymotrypsin, elastase, or a commercial enzyme preparation with a combination of enzymes) which are sensitive to pH and temperature. The most common industrial method for the production of WPH is in a batch reactor, under mild conditions (e.g., pH 6–8 and temperatures of 40–70°C, for 1–100 h), followed by heat treatment to inactivate the enzymes (Adler-Nissen, 1986).

Pre-hydrolysis heat-treatments can also have a significant effect on the physicochemical properties of whey protein hydrolysates; denaturation and unfolding of whey proteins has been shown to increase the rate of enzymatic hydrolysis by some enzymes due to the exposure of previously-buried peptide bonds which increases their susceptibility to proteolysis (Foegeding *et al.*, 2002). O’Loughlin *et al.* (2012)

reported that thermal treatment of WPI solutions (10%, w/v, protein) at pH 7.0, 75 °C for 5 min increased the rate of hydrolysis by ~45% when hydrolysed to a target degree of hydrolysis of 5% by the enzymatic preparation Corolase-PP, compared to the hydrolysis of unheated WPI. The DH of WPH is dictated by the application; WPH with a low to moderate DH (1-15%) can be used in easy-to-digest infant formulas (IF) for those infants that suffer from difficulty digesting intact proteins and sports nutritional products (Drapala *et al.*, 2016a,b; Nguyen *et al.*, 2015).

In addition, selectively hydrolysed whey protein ingredients have been developed recently, in which one major whey protein (e.g.,  $\beta$ -lg) is hydrolysed while the other major whey proteins (i.e.,  $\alpha$ -lac) remains intact resulting in protein ingredients with higher heat stability. Murphy *et al.* (2015) reported that when a whey protein ingredient in which  $\beta$ -lg was selectivity hydrolysed was incorporated into a model infant formula, that formula had a lower viscosity (14.8 mPa.s) than that of a model infant formula containing the corresponding intact whey protein ingredient (48.6 mPa.s) prior to spray drying (55% total solids). WPH with high DH (>~30%) can be used in hypoallergenic IF, where the presence of specific sequences of amino acids in whey proteins (e.g.,  $\beta$ -lg) that may potentially result in an allergenic response are hydrolysed. As an example, Nakamura *et al.* (1993) used two proteases to reduce the antigenicity of whey proteins 1000 fold. However, antigenic peptide structures may remain intact even after an extensive hydrolysis, in which case, membrane processing can be a solution as short peptides can be obtained in the permeate from ultrafiltration while long, antigenic sequences are retained (Guadix *et al.*, 2006).



**Fig. 1.9.** Schematic representation of the cleavage of peptide bond of proteins resulting in the liberation of amine and carboxylic acid groups.

Limited controlled hydrolysis of whey proteins can alter their physicochemical functionalities, including solubility, heat stability, viscosity, emulsifying and foaming properties (Singh & Dalgleish, 1998; Wijayanti *et al.*, 2014). However, hydrolysis of whey proteins may also increase their susceptibility to destabilisation when heated, due to the exposure of previously buried hydrophobic amino acids, reactive sites such as free -SH groups and/or the release of specific peptides that promote peptide-peptide and peptide-protein aggregation (Adjonu *et al.*, 2013; Creusot & Gruppen, 2007). A common challenge encountered during the preparation of foods (e.g., emulsions) that contain hydrolysates is their diminished processing stability and shelf life stability compared with formulations containing intact whey proteins (Drapala *et al.*, 2015, 2016a,b; Singh & Dalgleish, 1998; Ye & Singh, 2006).

## 1.4 Whey protein denaturation and aggregation

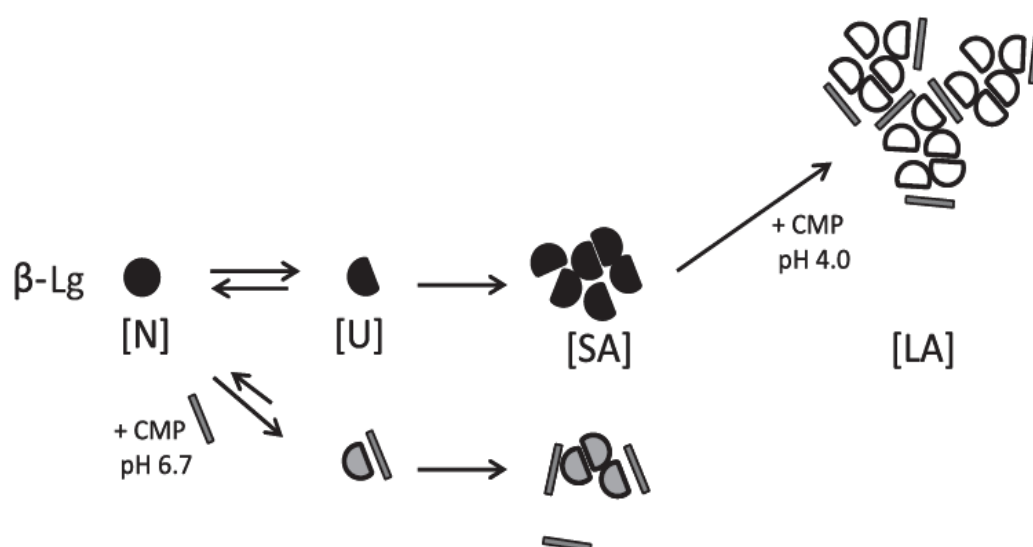
The thermal stability of whey proteins has been investigated for over 60 years and there are many detailed reports in the literature on denaturation and aggregation of whey proteins in numerous systems (Brodkorb *et al.*, 2016; Croguennec *et al.*, 2004; Donovan & Mulvihill, 1987; Havea *et al.*, 2009; Joyce *et al.*, 2016; Oldfield *et al.*, 2005; Ryan *et al.*, 2013; Sağlam *et al.*, 2014; Schmitt *et al.*, 2007). Heat-induced denaturation and aggregation of mixed whey protein systems involves a complex array of reactions with  $\beta$ -lg dominating the denaturation and aggregation process due to its higher concentration and reactivity than the other whey proteins. Nevertheless, closer investigations have revealed the importance of  $\alpha$ -lac, BSA and GMP in heat-induced denaturation and aggregation reactions in WPI solutions (De Wit, 1990; Havea *et al.*, 1998). For example, Croguennec *et al.* (2014) reported that smaller aggregates formed in GMP- $\beta$ -lg solutions on heating (pH 6.7, 65-95°C) compared to  $\beta$ -lg solutions heated without GMP, due to the high charge density of GMP protecting unfolded  $\beta$ -lg molecules from extensive covalent and non-covalent aggregation (Fig. 1.10). This highlights that whey protein ingredients (e.g., WPI) produced by different methods, such as membrane filtration or IEX as detailed in Section 1.3, would be expected to have different denaturation and aggregation kinetics.

There are inherent difficulties in relating denaturation/aggregation pathways of model whey protein systems to the complex, heterogeneous systems of commercial whey protein ingredients (Fig. 1.11). This is due mainly to the whey proteins possessing specific attributes (e.g., the number of Cys residues, disulphide bonds and free sulphydryl groups), as detailed in Section 1.2, that result in differences in their thermal stability; generally the thermal stability of whey proteins increases in the



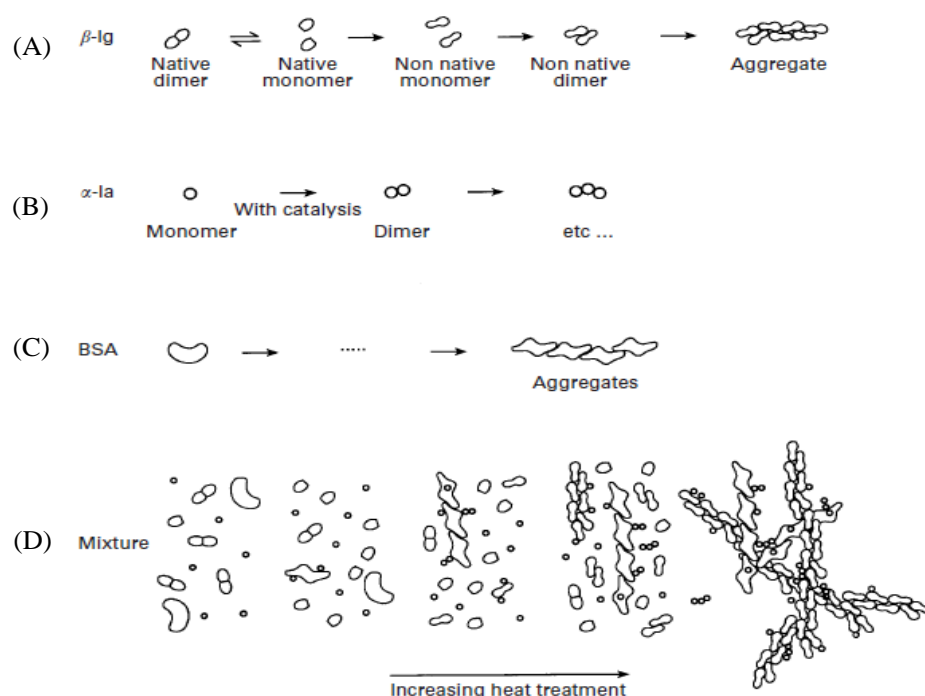
following order; immunoglobulins < BSA <  $\beta$ -lg <  $\alpha$ -lac < PP (Donovan & Mulvihill, 1987). The rate and extent of the denaturation is influenced by environmental factors as well as processing conditions with the reaction kinetics of whey proteins strongly dependent on the protein concentration. Protein concentration mainly affects the aggregation step rather than the unfolding of whey proteins and with increasing protein concentrations (10-40% protein), there is an increase in the formation of higher  $M_w$  aggregates (Dissanayake *et al.*, 2013; Verheul *et al.*, 1998; Wolz & Kulozik, 2015).

Fitzsimons *et al.* (2007) reported that the denaturation and aggregation processes in whey proteins can be separated into two separate stages where the first stage is denaturation (partial unfolding) of the native globular whey protein structure



**Fig. 1.10.** Schematic representation of how caseinomacropeptide i.e., glycomacropeptide (CMP) affects the denaturation/aggregation process of  $\beta$ -lactoglobulin ( $\beta$ -lg); N, native  $\beta$ -lg; U, unfolded  $\beta$ -Lg; SA, small aggregates; LA, large aggregates. Grey, negatively-charged molecules; white, positively-charged molecules (from Croguennec *et al.*, 2014)

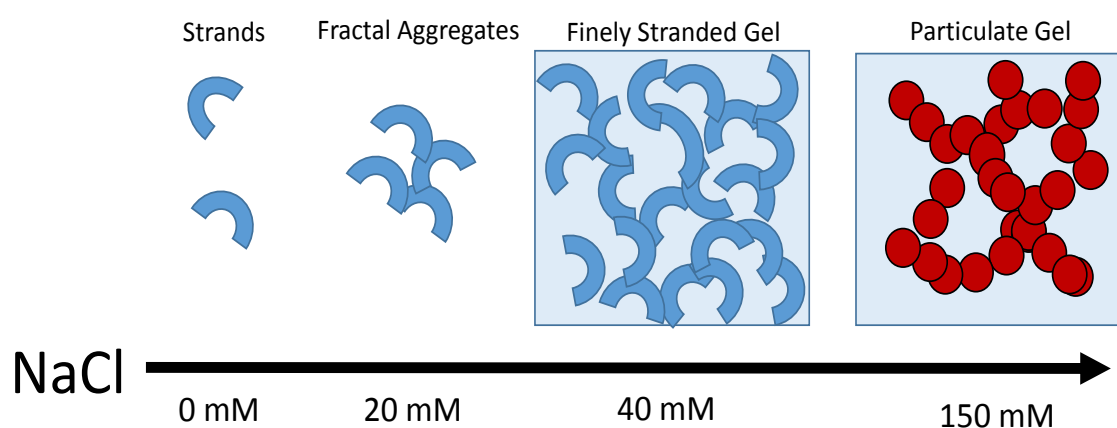
and the second stage is intermolecular aggregation of the denatured proteins. When heated at temperatures  $>70^{\circ}\text{C}$ , whey proteins unfold, potentially exposing free sulfhydryl (particularly for  $\beta$ -lg and BSA) and hydrophobic groups which would have been previously buried in the interior of their globular structure (Mulvihill & Donovan, 1987; Wijayanti *et al.*, 2014b). A dynamic equilibrium with the equilibrium constant between the protein in its native conformation and the partially-unfolded protein is reached and unfolding can be reversible where the protein molecules can refold to their native conformation if the temperature is reduced by rapid cooling below the unfolding temperature before aggregation has taken place (Croguennec *et al.*, 2003; Wolz & Kulozik, 2015). Under certain extrinsic conditions (e.g., pH, temperature, ionic strength), these denatured whey protein molecules/particles have



**Fig. 1.11.** Schematic representation of the formation of gel networks in heated ( $75^{\circ}\text{C}$ ) solutions of (A)  $\beta$ -lactoglobulin ( $\beta$ -lg), (B)  $\alpha$ -lactalbumin ( $\alpha$ -lac) (C) bovine serum albumin (BSA) and (D) mixtures of  $\beta$ -lg,  $\alpha$ -lac and BSA (from Havea *et al.*, 2001)

the ability to form irreversible intermediate aggregates *via* disulphide or sulfhydryl-disulphide reactions/interchange with more minor contributions from hydrophobic, Van der Waals and electrostatic interactions (Brodkorb *et al.*, 2016; Kehoe *et al.*, 2011). These intermediate whey protein aggregates can then further aggregate to form higher  $M_w$  aggregates (Mulvihill & Donovan, 1987; Wijayanti *et al.*, 2014b).

Changes in the pH of whey protein solutions can alter charge of the protein and electrostatic interactions along with promoting oxidation of the disulphide bonds (Brodkorb *et al.*, 2016; Monahan *et al.*, 1995). The pH of the solution and the nature of the agent used to adjust the pH (e.g., NaOH, KOH) also impacts the kinetics of the resultant denaturation and aggregation (Langton & Hermansson, 1992; Xiong *et al.*, 1993). Dissanayake *et al.* (2013) reported that there was a substantial increase in the rate of thermal denaturation and aggregation of whey proteins near their isoelectric point (pH 5.0) at relatively low (10%) protein concentration, whereas at higher protein concentrations (>17.5%, protein, w/v) and pH 4.0, whey proteins had a reduced rate of aggregation and greater structural stability. The presence of ions (e.g., monovalent salts) can alter the rate of whey protein aggregation during heat treatment, and also alter the size and shape of the aggregates formed (Fig. 1.12); this effect is due to a reduction in electrostatic repulsion between charged residues of denatured proteins due to charge-screening by ions, which promotes protein-protein interactions (Durand *et al.*, 2002). Monahan *et al.* (1995) reported that at > pH 9, significant SH-SH oxidation to thiol group occurred even at room temperature in a WPI solution which acted as an initiator of thiol/disulfide (SH/S-S) exchange reactions. In contrast, the total sulphdryl content of WPI solutions at pH 3 and 5 did not change on heating (>85°C), indicating that polymerization reactions involving SH/S-S interchange rather than thiol oxidation predominated. Estimation of the degree of unfolding of the whey proteins by measuring



**Fig. 1.12.** Schematic representation of the effect of adding NaCl on the structure of  $\beta$ -lg solutions (0.4% protein) at pH 7.0 after heating (60–85°C) (adapted from Phan-Xuan *et al.*, 2013)

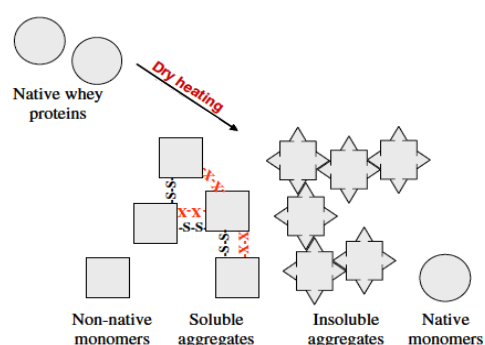
the exposure of hydrophobic amino acid residues showed that at pH 9 and 11 extensive irreversible unfolding of the protein molecules had occurred at room temperature. Methods to assess denaturation are based on principles which can be classified into six groups according to Kauzmann (1959): (1) thermodynamic, (2) optical properties or (3) chemical properties, (4) nuclear and electron magnetic resonance, (5) surface phenomena, and (6) rheology. Analytical tools used to detect and characterize protein aggregates can be broadly categorized as chromatographic (e.g., size exclusion chromatography), electrophoretic (e.g., sodium dodecyl sulphate-polyacrylamide gel electrophoresis), light scattering (e.g., dynamic light scattering), microscopic (transmission electron microscopy) and spectroscopic (free sulphydryl groups, surface hydrophobicity) methods (den Engelsman *et al.* 2011).

A limited number of techniques are available to measure the real-time formation of protein aggregates and nano-assemblies, especially those that provide information on both the size and the intrinsic properties of the aggregates at a broad range of temperatures and states (solution, suspension of protein nano-particles, and

gel). Taterka and Castillo (2015) reported a method that utilises an inexpensive/simple optical light backscatter system that monitors whey protein aggregation in real-time using changes in light backscatter signal.

Dry heating of proteins has gained a lot of interest in the pharmaceutical industry for viral and microbial decontamination of heat-sensitive products. Controlled dry heating has now become an established industrial process for improving the functional properties of food proteins such as egg proteins (Kato *et al.*, 2003). Structural changes in proteins on dry heating cannot be related to kinetic results obtained for heating whey protein in solution (Povey *et al.*, 2009) and only a limited number of studies have reported on the impact of dry heating of whey proteins at temperatures <50°C on their functional properties (Gulzar *et al.*, 2012 and references therein; Norwood *et al.*, 2016). Gulzar *et al.* (2011) reported that dry heating of WPI (initial pH of 6.5, at 23% RH, at 100°C for up to 24 h) resulted in the formation of soluble and insoluble protein aggregates formed *via* covalent cross-links and disulphide bonding (Fig. 1.13).

The presence of carbohydrates in whey protein solutions has been shown to modify the kinetics of whey protein denaturation, which can be partially attributed to macromolecular crowding in the solution which can limit protein unfolding (Garrett *et al.*, 1986; Kulmyrzaev *et al.*, 2000; Zhu *et al.*, 2008). Conjugation of whey proteins has also shown to increase their denaturation temperature;

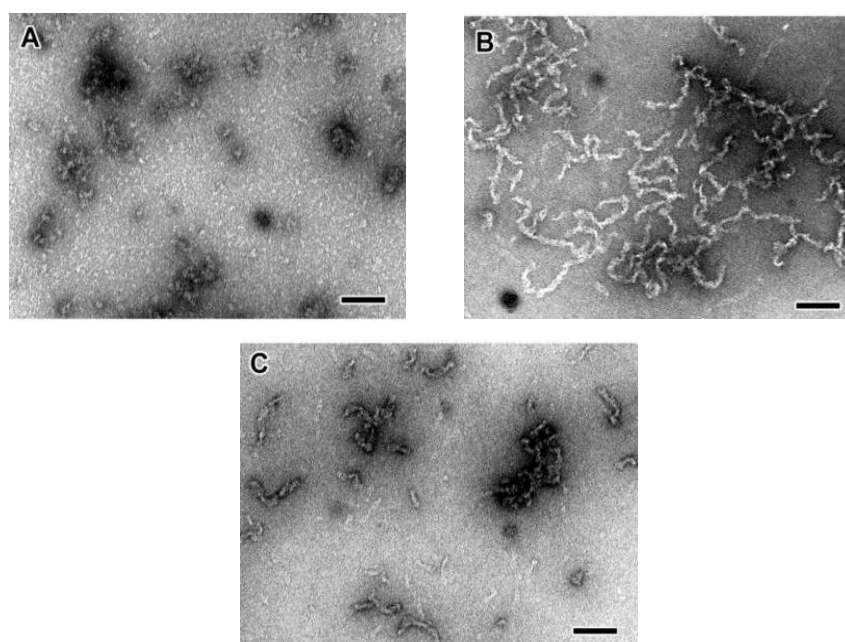


**Fig. 1.13.** Proposed mechanism for how dry heating modulates the denaturation/aggregation mechanism of whey proteins.  $-S-S-$ , disulphide bond;  $-X-X-$ , covalent bonds other than disulphide bond (from Gulzar *et al.*, 2012).

Mulsow *et al.* (2009) reported that WPC (60% protein, 30% lactose) dry heated at 60°C for 96 h subsequently had an increased denaturation temperature (~10°C increase) compared to the native WPC, which was attributed to increased hydrophilicity due to covalently attached lactose which in turn inhibited hydrophobic interactions between whey protein molecules.

Furthermore, conjugation of whey protein can also impact the structure of heat-mediated whey protein aggregates; Pinto *et al.* (2011; 2014) reported that conjugation of  $\beta$ -lg with glucose by heating at pH 7.0, at 90 °C for 4-24 h, slowed down the rate of heat-induced aggregation and short fibrils and polydisperse aggregates formed compared to  $\beta$ -lg heated alone where covalently-linked fibrillar aggregates were formed (Fig. 1.14). This was attributed to the presence of the glucose in addition to the  $\beta$ -lg in solution, which hindered the growth of covalent aggregates, but not the initial denaturation step. Several approaches have been suggested to control aggregation of whey protein including conjugation of the proteins with carbohydrate, the addition of hydrophobic compounds prior to heating by changing the equilibrium of unfolding of  $\beta$ -lg by maintaining the protein in the native conformation state at

higher temperatures (e.g., hydrolysed/hydroxylated lecithin, and saturated/unsaturated fatty acids), cross-linking whey protein with transglutaminase and modifying the level of divalent cations (e.g.,  $\text{Ca}^{2+}$ ) in the protein solution (Guyomarc'h *et al.*, 2015; Joyce *et al.*, 2016; Wijayanti *et al.*, 2014b). Another approach to control whey protein aggregation adopted by Wijayanti *et al.* (2014a) and Croguennec *et al.* (2003) was to inhibit some of the disulphide or sulfhydryl-disulphide mediated interactions between whey proteins using reducing or thiol blocking agents such as dithiothreitol (DTT), *N*-ethylmaleimide (NEM),  $\beta$ -mercaptoethanol and dihydrolipoic acid (DHLA).



**Fig. 1.14.** Transmission electron microscopy images of negatively stained native  $\beta$ -Lg (A); heated for 4 h at 90 °C without (B) and with (C) glucose. Bars = 100 nm (from Pinto *et al.*, 2011)

## 1.5 Protein-carbohydrate conjugation

A conjugated protein is defined as a protein to which another chemical group (e.g., carbohydrate) is attached by either covalent bonding or other interactions (Wong, 1991). Milk proteins and peptides, in the presence of reducing carbohydrates, can undergo a series of complex chemical changes during heating, known as the Maillard reaction. Conjugation occurs naturally during the early stages of the Maillard reaction when a covalent bond forms between the protein and carbohydrate components, resulting in the release of water (i.e., condensation reaction). The resulting covalently-linked Schiff base product can undergo irreversible Amadori rearrangement, leading to the formation of Amadori products (Ames, 1992; Liu *et al.*, 2012; Zhu *et al.*, 2008). Conjugation of food proteins with carbohydrates *via* the Maillard reaction is a growing area of interest, with many studies completed, particularly over the last 10-15 years, on the use of conjugation to modify physicochemical and functional properties of proteins and peptides (Fig. 1.15).

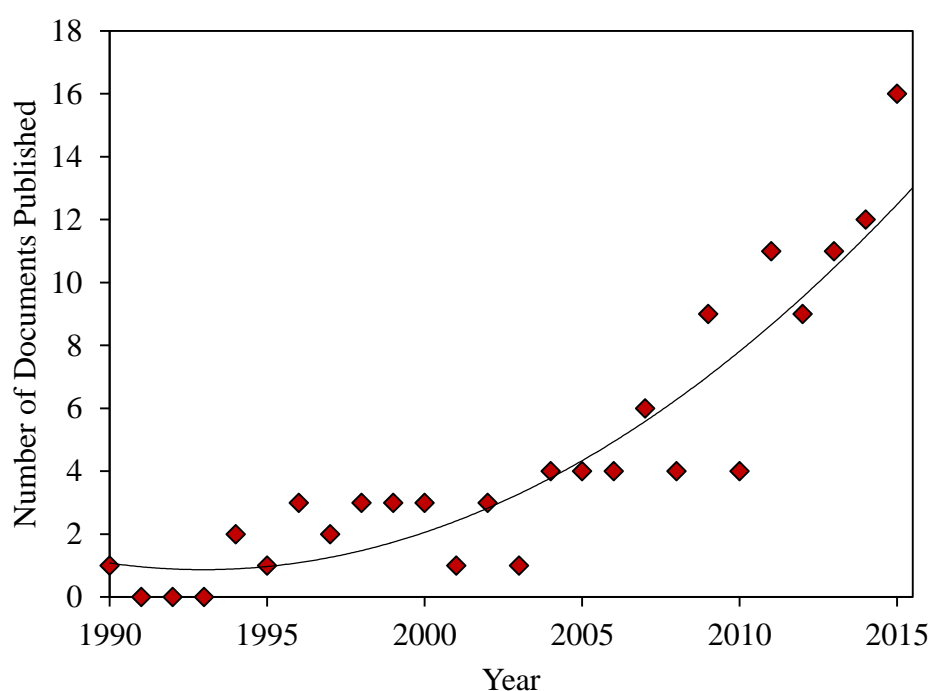
In the food industry, the principal technological hurdles limiting the use of whey protein ingredients in the formulation of value-added beverages and powders are, (1) poor solubility of intact proteins in high acid ready-to-drink beverages, resulting in the development of turbidity and phase separation (Akhtar & Dickinson, 2007), (2) poor emulsification properties of hydrolysed proteins (Singh & Dalgleish, 1998; Agboola *et al.*, 1998a,b), causing challenges with emulsion formation, stabilisation and spray drying (e.g., powder stickiness and high free fat) during the manufacture of powdered nutritional products, and (3) physical instability such as aggregation, sedimentation and creaming during processing and shelf-life in high ionic strength environments and during thermal processing (Yadav *et al.*, 2010).



Conjugation has been shown to be successful in modifying the functional properties of a range of milk protein/peptide-based ingredients.

## 1.6 The Maillard reaction

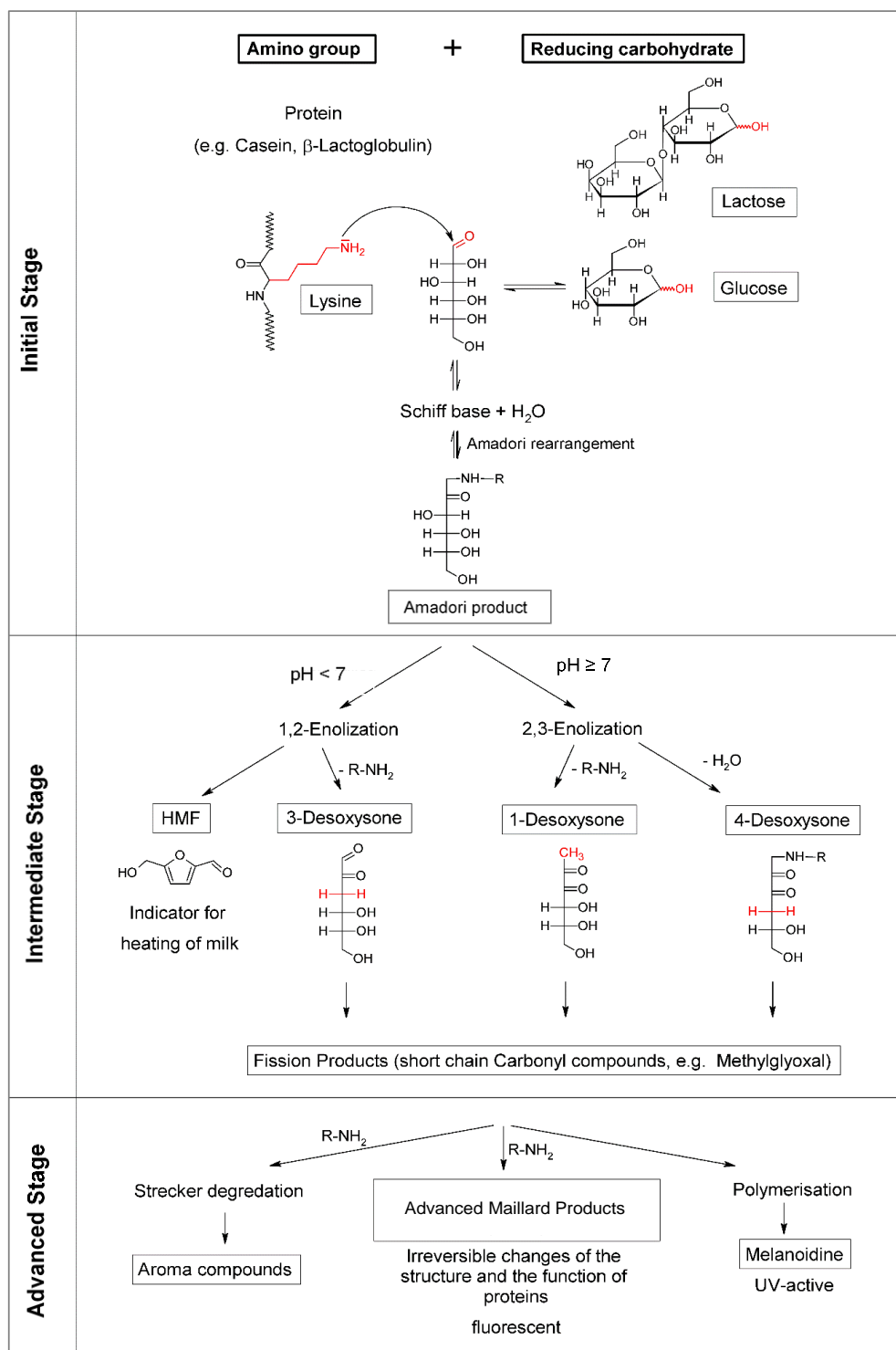
The Maillard reaction (Maillard, 1912) encompasses a complex series of reaction pathways, many of which proceed concurrently during heating and/or storage of protein/carbohydrate mixtures. Understanding of the complexity of the Maillard reaction has been advancing steadily over the years, and a brief overview is provided here in the context of the Maillard reaction being the main mechanism by which milk proteins and peptides are conjugated with carbohydrate molecules.



**Fig. 1.15.** The number of peer-reviewed reviews and papers published each year, over the last 25 years as captured using the search query “whey protein” and “conjugation” and/or “glycation” in food, agriculture and chemistry journals (from Scopus, 2016)

Hodge (1953) was the first to develop a simplified, integrated scheme for the Maillard reaction, which has been advanced further and refined by researchers from different fields over the years (Henle *et al.*, 1991; Van Boekel, 1998; Zhang & Zhang, 2007). In essence, Hodge (1953) divided the chemistry of the Maillard reaction into three stages - the early, intermediate and advanced stages (Fig. 1.16). The early stage of the Maillard reaction involves a series of individual reactions that are initiated when the  $\epsilon$ -amino groups of lysine and the  $\alpha$ -amino groups of terminal amino acids in proteins/peptides condense with the carbonyl groups of reducing carbohydrates, to form a Schiff base, with the release of a molecule of water (Ames, 1992). Proteins only have one  $\alpha$ -amino group, but numerous  $\epsilon$ -amino groups. However, the higher pKa renders the lysyl side chains effectively less nucleophilic; thus the  $\alpha$ -amino group is more reactive than the  $\epsilon$ -amino groups. The Schiff base is thermodynamically unstable and undergoes spontaneous rearrangement to form either an Amadori (in the case of aldoses) or Heyn's (in the case of ketoses) product (Wrodnigg & Eder, 2001).

The intermediate stage of the Maillard reaction involves the degradation of the Amadori and/or Heyn's rearrangement products by a number of different reactions, including cyclisations, dehydrations, retro-aldolisations, isomerisations and further condensations, which causes degradation of amino acids and carbohydrates (Ames, 1998). The advanced stages are complex and variable, depend on the reaction conditions, and involve dehydration and decomposition of the early reaction products, resulting in the production of many advanced Maillard reaction products (AMP) and coloured nitrogenous polymers and co-polymers, known collectively as melanoidins (Ames, 1998; Hodge, 1953).



*Fig. 1.16. Simplified overview of the Maillard reaction in milk and milk products (based on Hodge, 1953; Ames 1998).*

While, from a functionality perspective, it is desirable to achieve conjugation in the early stages of the Maillard reaction, it is normally desirable to limit the progression of the Maillard reaction to advanced stages, as AMP's are largely responsible for some of the less desirable consequences of the Maillard reaction, e.g., generation of off-flavours, loss of nutritional value, protein crosslinking and generation of potentially toxic compounds (Uribarri *et al.*, 2005). Many analytical approaches have been reported for monitoring the formation of Maillard reaction products and for determining the progression of the Maillard reaction through the early, intermediate and late stages. An overview of the analytical approaches most commonly used in protein/peptide-carbohydrate conjugation studies is provided in Table 1.4.

## **1.7 Factors affecting Maillard-induced conjugation**

In the production of protein/peptide-carbohydrate conjugates, the rate, extent and course of the Maillard reaction are influenced by several intrinsic and extrinsic factors, including, but not limited to, nature of the reactants, temperature, time, pH and water activity ( $a_w$ ) (Ames, 1990; de Oliveira *et al.*, 2016; Liu *et al.*, 2012; Oliver *et al.*, 2006a; Van Boekel, 2001). Understanding and manipulation of these factors allow control of the yield, quality and functionality of conjugated proteins/peptides.

### *1.7.1 Nature of the reactants*

The physicochemical properties (i.e., molecular weight,  $M_w$ ; structure/conformation and surface charge) of the amino and carbonyl compounds, and their molar ratios, all govern the rate and extent of the Maillard reaction, and consequently, the physicochemical properties of the conjugated proteins/peptides.

Reactivity of compounds tends to decrease with increasing  $M_w$ , due to the greater contribution of steric hindrance with increasing  $M_w$ ; as an example, monosaccharides are more reactive with proteins than di- or oligosaccharides under conditions which favour conjugation. For protein hydrolysates, the degree of hydrolysis,  $M_w$  profile and charge of the peptides are important in determining their reactivity during Maillard-induced conjugation (Drapala *et al.*, 2016a,b; Mulcahy, *et al.* 2016b, Chapter 5; Van Lancker *et al.*, 2011).

**Table 1.4.** An overview of the analytical approaches most commonly used in protein/peptide-carbohydrate conjugation studies (adapted from O'Brien, 1997)

Stage	Measurement	Reference
Early	Reduction in chemically reactive amino groups including 1-fluoro-2,4-dinitrobenzene (FDNB), trinitrobenzenesulfonic acid (TNBS), guanidination, sodium borohydride, <i>o</i> -phthalaldehyde (OPA) and dye-binding methods.	Mehta & Deeth (2016)
	Formation of lactulose-lysine	Henle <i>et al.</i> (1991)
	Absorbance of Schiff base	Zhu <i>et al.</i> (2008)
	HPLC analysis of derived Amadori products (i.e., furosine, carboxymethyllysine)	Erbersdobler & Somoza (2007)
	Amino acid analysis	Rutherford <i>et al.</i> (2012)
Intermediate	Identification of conjugation sites by electrospray ionization or matrix assisted laser desorption/ionization mass spectrometry	Oliver (2011)
	HPLC analysis of intermediate reaction products or their derivatives (e.g., hydroxymethylfurfural)	Morales <i>et al.</i> (1995)
Advanced	pH decrease	Liu <i>et al.</i> (2008)
	Colour development (absorbance or colorimeter)	Ames (1998)
	Fluorescence of advanced Maillard products	Birlouez-Aragon <i>et al.</i> (1998)
	Strecker degradation volatiles by gas chromatography-mass spectrometry/flame ionization	Jansson <i>et al.</i> (2014)
	Enzyme-linked immunosorbent assay	Horiuchi <i>et al.</i> (1991)

### *1.7.2 Temperature and Time*

Louis-Camille Maillard (Maillard, 1912) was the first to report that the rate of the Maillard reaction increased with increasing temperature and duration of heating. In addition, more recent research has shown that temperature also affects the nature (e.g., conformation and accessibility to reactive protein/peptide functional groups) of the reactants. The reactivity of sugars increases with increasing temperature as the proportion of reducing sugar molecules present in the open-chain form (i.e., the more reactive form) increases, due in part to the faster rate of mutarotation of the sugar molecules (Van Boekel, 2001). Heat-induced structural/conformational changes (e.g., denaturation and aggregation) of milk proteins/peptides may result in amino groups becoming less available for participation in the Maillard reaction (Chevalier *et al.*, 2001; Jiang & Brodkorb, 2012; Mehta & Deeth, 2016).

### *1.7.3 pH*

pH influences the reactivity of both the carbohydrate and protein components of mixtures during the Maillard reaction; a basic environment can catalyse the initial stages of the Maillard reaction by deprotonating the amino groups, which in turn increases reactivity with carbonyl groups of reducing carbohydrates. The open chain form of the carbohydrate and the un-protonated form of the amino group, which are considered to be the most reactive forms, are usually favoured at higher pH, up to a maximum of pH ~9-10 (Martins *et al.*, 2000).

The use of buffers, such as phosphate, phthalate and acetate, to help minimise changes in pH on heating, have been reported to promote the Maillard reaction, as

measured by loss of available amino groups and development of brown colour (Bell, 1997; Van Boekel, 2001). For example, at a constant pH between 5 and 7, the presence of phosphate buffer has been shown to increase the rate of the Maillard reaction by ~15 fold compared to a non-buffered system, as the phosphate acts as an acid-base catalyst during the Amadori rearrangement (Potman & Van Wijk, 1989).

The pH of protein/peptide-carbohydrate mixtures can decrease (depending on the buffering capacity) as the Maillard reaction progresses due to the formation of acids (e.g., formic and acetic acids), the consumption of acidic amino groups (e.g., lysine) or the loss of carboxyl groups during Strecker degradation, resulting in the production of carbon dioxide (Nursten, 2005). Furthermore, products derived from the intermediate and advanced stages of the Maillard reaction are degraded by different reaction pathways depending on the pH of the system. For example, degradation of the Amadori products at  $\text{pH} \leq 7$  takes place *via* the 1, 2-enolisation pathway that favours the formation of furfural or hydroxymethylfurfural (HMF), whereas, at  $\text{pH} > 7$ , the degradation of Amadori products proceeds through the 2, 3-enolisation pathway, favouring the production of reductones and fragmentation products such as hydroxyacetone and 2, 3-butanedione, minimising the formation of HMF (Ames, 1998; Liu *et al.*, 2012).

#### *1.7.4 Water activity/relative humidity*

Increasing water activity of protein/peptide-carbohydrate mixtures generally increases the rate and extent of conjugation, due to the increased diffusion and mobility of reactants; however, high water concentrations/ $a_w$  can negatively influence progression of the Maillard reaction. Morgan *et al.* (1999b) reported that, when heated



at the same temperature, the rate and extent of protein-carbohydrate conjugation in an aqueous solution was lower than when a dry heating approach was used, as the presence of water inhibits the initial Amadori condensation reaction between the available amino and carbonyl groups. The physicochemical state of the reactants in mixtures of proteins, peptides and carbohydrates can also influence the progression of Maillard-induced conjugation; when reactants (e.g., sugars) in the amorphous state are exposed to high humidity ( $a_w$ ) they generally absorb water until the reactant molecules acquire sufficient mobility (generally at  $a_w \sim 0.6-0.7$ ) and space to form a crystalline lattice. On crystallisation of sugars, water is released and may become trapped in a localized manner within protein/peptide-carbohydrate mixtures, and facilitate further interactions/conjugation between proteins/peptides and amorphous sugars (Lievonon *et al.*, 1998; Lievonon *et al.*, 2002; Roos *et al.*, 1996).

#### *1.7.5 Other factors*

Factors, other than those outlined above, can impact the Maillard reaction, including the presence of sulphur dioxide in food systems which has been shown to delay the development of brown colour (Ames, 1990) and the presence of metal ions, which can accelerate or inhibit the Maillard reaction, depending on their concentration (Ramonaitytė *et al.*, 2009). In model systems, the presence of tertiary amine salts, acetic acid and free radicals have been shown to promote the Maillard reaction; however, these factors may often, in practice, be of minor significance relative to the nature of the reactants, temperature, time and moisture content (O'Brien, 1997). Alternative treatments (e.g., ionizing radiation, pulsed electric field, microwave irradiation, ultrasound treatment) have also been shown to produce Maillard reaction

products, including brown pigments and volatile flavour compounds (Corzo-Martínez *et al.*, 2014; Guan *et al.*, 2006, 2010; Shi *et al.*, 2010).

## **1.8 Protein/peptide substrates used in conjugation**

The conjugation of milk proteins/peptides has been studied using many categories of milk protein-based ingredients as substrates, including, but not limited to, WPC and WPI, individual whey protein fractions (in particular  $\beta$ -lg,  $\alpha$ -lac and BSA), sodium caseinate, casein fractions ( $\beta$ -casein) and hydrolysates of whey proteins (WPH) and caseins (e.g., hydrolysed sodium caseinate). For the reasons outlined later in Section 1.11, it is desirable that the proteins/peptides used are soluble under the conditions of conjugation; hence only soluble forms of casein (e.g., sodium caseinate) have been studied. It is also desirable that the proteins used for conjugation are present in a conformation which ensures a high degree of accessibility of carbonyl groups to amino groups, which is one of the main reasons why, in the study of casein-based conjugates, sodium caseinate, with an open/flexible structure, and extremely low levels of non-protein components, has been extensively used as the casein protein substrate; the authors are not aware of any studies performed using micellar casein for production of casein-based conjugates.

Whey proteins are more susceptible than caseins to heat-induced aggregation under the conditions used for conjugation (particularly under wet heating conditions), which would be expected to restrict accessibility of carbonyl groups to amino groups on the protein/peptide molecules. It is desirable to have low levels of non-protein components (e.g., lactose, minerals and lipid) and non-protein nitrogen components (e.g., ammonia) in the protein-containing ingredients used as substrates for

conjugation, as lactose and ammonia contributes strongly to brown colour and flavour compound formation (Lillard *et al.*, 2009), minerals promote aggregation of whey proteins (Brodkorb *et al.*, 2016), lipid material can contribute to off-flavour formation (Liu & Zhong, 2014; Lloyd *et al.*, 2009) and lactose can compete with other carbohydrates for conjugation to the protein substrate during heating under conditions required to achieve conjugation. Therefore, high protein content WPC and WPI, or pure protein fraction ingredients are most commonly used for conjugation purposes.

Hydrolysis of casein and whey protein molecules increases the number of free amino groups available to react with carbonyl groups during conjugation and can also lead to increased exposure and accessibility to previously-buried lysine residues. Hydrolysis of whey proteins, due to reduction of average  $M_w$  and levels of secondary structure, enhances their stability to heat-induced aggregation, which can facilitate enhanced retention of amino groups in a form accessible for conjugation during heating. For example, Ju *et al.* (1995) reported that limited hydrolysis of WPI (DH 2-7%), using trypsin, prevented heat-induced gelation of a WPI solution (12%, w/v, protein) on heating at 80°C for 30 min at pH 3 and 7. Mulcahy *et al.* (2016b) reported that WPH with a low degree of hydrolysis (DH 9.3%) had 55.4% higher levels of available amino groups compared with an intact WPI counterpart, which contributed to more rapid and extensive conjugation of maltodextrin (MD) with the WPH than with the WPI.

## **1.9 Carbohydrate substrates used in conjugation**

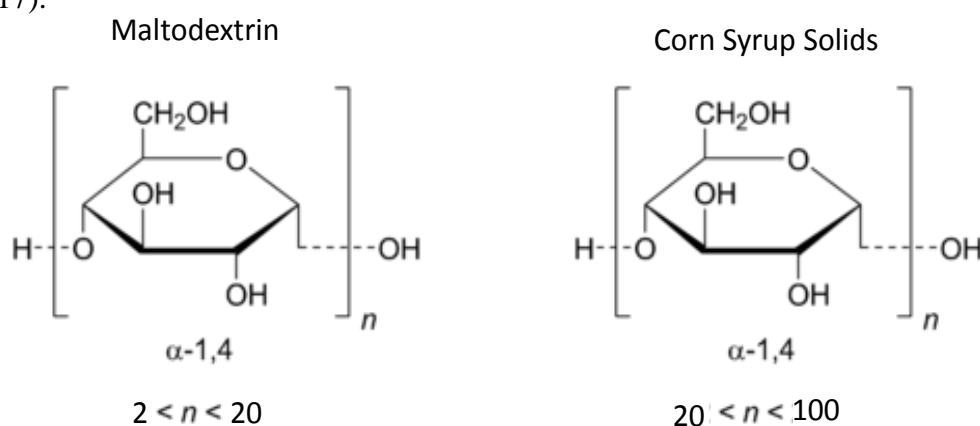
In order to participate in the Maillard reaction the carbohydrate must be capable of acting as a reducing agent, i.e., have an available aldehyde group or ketone

group. All monosaccharides are reducing sugars, along with some disaccharides, oligosaccharides, and polysaccharides. From the point of view of their ability to participate in Maillard-induced conjugation of milk proteins/peptides, and the functionality of the resultant conjugates, the key differences between these carbohydrates are chain length, structure (i.e., linear vs branched and ketoses vs aldoses) and charge (neutral vs charged). Conjugation of milk proteins/peptides has been studied using many different types of carbohydrate ingredients, including, but not limited to, lactose, dextrans, glucose, maltose, ribose, guar gum, pectin, fenugreek gum, oligosaccharides, glucosamine, maltodextrin (MD) and corn syrup solids (CSS).

In general, the shorter the chain length of the carbohydrate component, the faster the rate, and the greater the extent of conjugation. On conjugation of whey protein with MD or CSS, having dextrose equivalent (DE) values in the range 6-38, at an initial pH 8.2, at 90°C for up to 24 h, the extent of conjugation increased with increasing DE value of the MD and CSS ingredients (Mulcahy *et al.*, 2016a; Chapter 2). Delahaije *et al.* (2013) studied the stability of emulsions of patatin conjugated to the same extent with different mono- and oligosaccharides (xylose, glucose, maltotriose and maltopentaose) and reported that attachment of monosaccharides did not affect the flocculation behaviour of the emulsion; however, the attachment of maltotriose and maltopentaose ( $M_w > 0.5$  kDa) provided stability against flocculation of the emulsions at pH 5, due to increased steric stabilization contributed by the higher  $M_w$  carbohydrates. Brands and van Boekel (2001) reported that ketoses degraded during heating, whereas aldoses were involved in formation of the covalent bond between proteins and carbohydrates during the Amadori stage of the Maillard reaction.

### 1.9.1 Starch hydrolysis products

Starch hydrolysis polymers such as MD and CSS have potential to be used as sources of reducing carbohydrates during protein-carbohydrate conjugation as they are used extensively in the food industry as stabilisers, viscosity modifiers, energy sources, bulking agents, spray drying aids and sweeteners (Chen & O'Mahony, 2016). They consist of D-glucose units linked by  $\alpha$ -(1 $\rightarrow$ 4) and  $\alpha$ -(1 $\rightarrow$ 6) glycosidic bonds in chains of variable length and are classified according to their dextrose equivalent (DE) value which is a measure of the extent of starch hydrolysis and is usually determined by titration and a measure of the total reducing power of the sugars present relative to a dextrose (D-glucose) standard, on a dry mass basis. The DE value is inversely related to the number of monosaccharide units in a polysaccharide, i.e., the degree of polymerization (DP). For example, glucose has a DE value of 100, while intact starch has a nominal DE of zero (Sun *et al.*, 2010). MDs are starch hydrolysis products having DE values <20, while CSS have DE values of >20 (Pancoast & Junk, 1980; Fig. 1.17).



**Fig. 1.17.** Maltodextrin and corn syrup solids consists of D-glucose units connected in chains of variable length. The glucose units are primarily linked with  $\alpha$  (1 $\rightarrow$ 4) glycosidic bonds (from Pancoast & Junk, 1980).

Starch hydrolysis products such as MD and CSS are typically composed of a mixture of sugar molecules with different chain lengths by virtue of the hydrolysis process, with variations in chain length related to their DE value, i.e., the shorter the average chain length, the higher the DE value (Dziedzic & Kearsley, 1995). Starch hydrolysis products provide many benefits in the formulation of protein-carbohydrate beverages; for example, on an equal contribution to caloric density, high chain length glucose polymers (e.g., maltodextrins) contribute to lower osmolality, sweetness and cost, compared with low chain length glucose polymers (e.g., glucose) (Marchal *et al.*, 1999) (Table. 1.5). Commercial starches are obtained from corn, waxy corn, high amylose corn, wheat, rice and tubers. As starches are produced from different sources, different processing methods are used to isolate and enrich starches. The processes, conditions and type of starch used as the starting material will affect the composition, structure and functionality of the resulting starch hydrolysis products.

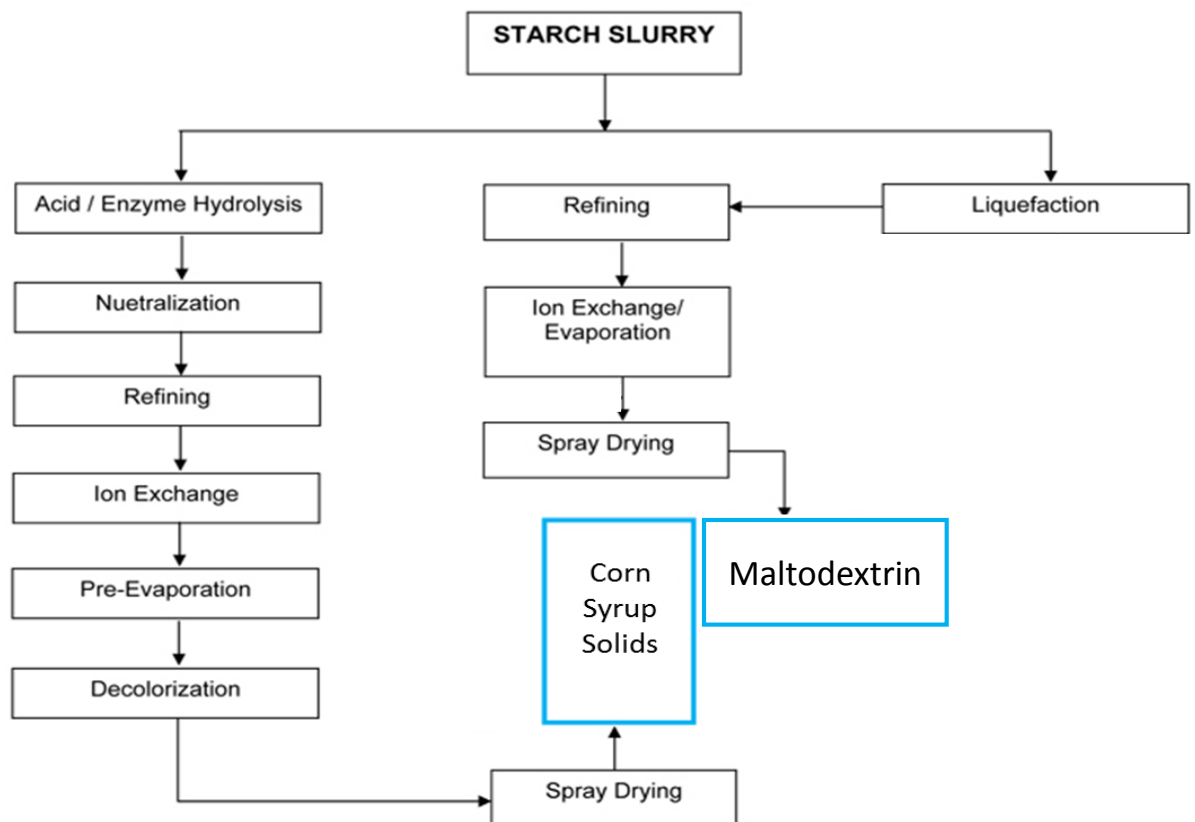
**Table. 1.5.** Typical properties of starch hydrolysis products as a function of dextrose equivalent value (from Hobbs, 2009).

<i>Property or functionality that increases with an increasing DE value</i>	<i>Property or functionality that decreases with an increasing DE value</i>
Ability to participate in the Maillard reaction	Viscosity
Flavour enhancement	Cohesiveness
Fermentability	Foam stabilization
Freezing point depression	Prevention of sucrose crystallization
Hygroscopicity	
Osmotic pressure	
Sweetness	

Starch occurs in the presence of other plant constituents and needs to be separated by exploiting the size (2-100  $\mu\text{m}$ ) and high density (1600  $\text{kg/m}^3$ ) of the starch granules (Rapaille & Van Hemelrijck, 1997). Native starches have limited functionalities in food applications and are usually physically, chemically or biologically modified by treatments such as molecular chain crosslinking, oxidation, esterification, acid and/or enzymatic hydrolysis to extend their potential food applications (Murphy, 2002). Starch hydrolysis products can be manufactured by either acid-, enzyme-, or a combined acid-enzyme processes. Acid hydrolysis of starch is less commonly used as acid conversion of starch results in a high amount of linear amylose that may slowly reassociate to form insoluble compounds (i.e., retrogradation) that result in the development of turbidity (Galliard, 1987). For example, starch hydrolysis products produced from waxy maize have a lower tendency to haze as they are composed almost entirely of branched amylopectin (Shavakhi *et al.*, 2012).

Native starch granules are semi-crystalline in structure, consisting of linear amylose (22-28%) and branched amylopectin (72-78%), which are resistant to hydrolysis by amylases (Galliard, 1987). When gelatinised (i.e., liquefaction), starch is readily enzymatically hydrolysed with  $\alpha$ -amylase and converted to glucose sub-units/molecules, thereby reducing the molecular weight of starch molecules. Enzymatic hydrolysis with  $\alpha$ -amylase efficiently hydrolyses the  $\alpha$ -(1 $\rightarrow$ 4) linkages between the glucose molecules, but not the  $\alpha$ -(1 $\rightarrow$ 6) linkages, leaving behind a small amount of high  $M_w$  residues before evaporation and spray drying (Fig. 1.18). During acid and enzyme hydrolysis, both  $\alpha$ -(1 $\rightarrow$ 4) and  $\alpha$ -(1 $\rightarrow$ 6) linkages are cleaved,

converting the starch molecule to increasingly lower  $M_w$  products (e.g., corn syrup solids).



**Fig. 1.18.** General process flow for manufacture of starch hydrolysis products such as maltodextrin and corn syrup solids (from <http://www.a2zstarch.com/>).



## **1.10 Mode of Conjugation**

As detailed in Section 1.7, the main variables that can be controlled during conjugation of milk proteins/peptides are temperature, time, pH, moisture content, relative humidity (RH) and/or  $a_w$ . These variables can be grouped to give 2 distinct approaches for achieving conjugation – (1) wet heating and (2) dry heating. The wet heating approach normally involves incubation of an aqueous solution of protein/peptide and carbohydrate reactants, commonly pre-adjusted to a target pH (normally pH 6.0-11.0), for a pre-determined time (e.g., min-days) at a set temperature (typically in the range 60-95°C). The conjugation reaction is normally stopped (or slowed considerably) by cooling and further processing (e.g., freeze or spray drying) of the conjugated protein/peptide solution. The dry heating approach normally involves incubation of a co-dried mixture (commonly pre-adjusted to a target pH) of the protein/peptide and carbohydrate ingredients for a pre-determined time (min-days) at a set temperature (typically in the range 60-130°C) at a set RH (typically 60-80%).

Both approaches have been used extensively for conjugation of milk proteins/peptides and both have their advantages and limitations. The mobility of reactants is higher with the wet heating than the dry heating approach and higher temperatures (for shorter times) are generally used with the former than with the latter; however, some recent studies have used considerably higher temperature (130°C) and shorter times (<30 min) than previous studies to achieve conjugation of WPI with lactose or MD under dry heating conditions at 79% RH (Liu & Zhong, 2014). Similarly, Guo and Xiong (2013) reported that WPI was successfully conjugated with lactose or MD (DE18) at 130°C for 20 min and 79% RH, with both systems having less colour development than WPI-lactose/MD conjugated at 80°C for 2 h.

To achieve maximum reactivity between the protein/peptide and carbohydrate components using dry heating, it is necessary to prepare a solution of the two components, which is dried before being conjugated by dry heating, and the conjugated powder typically requires down-stream drying due to release of water during the early stages of the Maillard reaction. During dry-heating, the release of water, due to the initial condensation reaction between available amino groups of proteins and carbonyl groups of carbohydrates, may lead to localised browning of the powdered reaction mixture during conjugation. Furthermore, sugar crystallisation of the carbohydrate moiety (e.g., lactose crystallisation) may also cause the release of water and resulting in localised increases in mobility of the reactants (Lievonon *et al.*, 1998), which is not an issue with the wet heating approach. While most of the research published to date using the wet heating approach has been conducted at temperature ranging from 60-95°C for time periods of minutes-days (Chevalier *et al.*, 2001; Darewicz, & Dziuba, 2001; Drapala *et al.*, 2016a,b; Morgan *et al.*, 1998; Mulcahy *et al.*, 2016a,b, Chapter 2, 5; Zhang *et al.*, 2012; Zhu *et al.*, 2008), some studies have reported the use of higher temperatures (i.e., 100-130°C) for shorter times ( $\leq 6$  h) to induce conjugation using wet heating; for example, Chen *et al.* (2013b) reported that phosvitin and dextran were conjugated by heating in an aqueous solution at 100°C for 6 h.

In addition to the differences in energy costs and efficiency between wet and dry heating approaches, the use of dry heating at lower temperatures ( $< 70^\circ\text{C}$ ) has been shown to result in greater preservation of the native 3-dimensional structure of whey proteins, compared with wet heating approaches, which has important implications for selected functional properties, such as solubility and interfacial properties (Gauthier

*et al.*, 2001; Morgan *et al.*, 1998; 1999a, b). The use of macromolecular crowding to effectively restrict denaturation and, in particular, aggregation of whey proteins has also shown promise on conjugation of WPI with dextran (Ellis, 2001; Perusko *et al.*, 2015; Zhu *et al.*, 2008).

## **1.11 Functional properties of protein-carbohydrate conjugates**

### *1.11.1 Solubility*

Milk proteins used in food products are generally required to have high solubility in order to facilitate expression of the desired functional properties such as gelation, aeration, water-binding, foaming and emulsification (de Wit, 1989; O'Regan *et al.*, 2009a). Solubility of milk proteins is influenced by many physicochemical properties of the protein molecules themselves, i.e.,  $M_w$ , conformation (e.g., as affected by denaturation/aggregation), amino acid composition, physical state, exposure of selected functional groups, surface hydrophobicity, and environmental factors, i.e., pH, temperature, ionic strength and nature of the solvent (De Wit & Klarenbeek, 1984; Hayakawa & Nakai, 1985; Vojdani, 1996).

Protein-carbohydrate conjugation *via* the Maillard reaction has been shown to be an effective means of increasing the solubility of milk proteins. Sodium caseinate has very different functionality to whey proteins (i.e., high viscosity at low concentrations and poor solubility at pH ~4.6) and is used as an emulsifier, texturizer and stabilizer in food products such as cured meats, processed cheese, coffee whiteners, high fat powders, bakery and confectionary products (Carr & Golding, 2016; O'Regan and Mulvihill, 2011; Swaisgood, 1993).

Improvements in the solubility of sodium caseinate at its isoelectric point would be expected to help broaden its application in food products; O'Regan and Mulvihill (2009b) reported that sodium caseinate conjugated with MD, with DE values of 4 or 10, had improved protein solubility (~5-90% increase) in the pH range 4.0-5.5, compared to sodium caseinate, particularly around the isoelectric point (~pH 4.6) of the protein. This increase in protein solubility on conjugation was attributed to an increase in the hydration of the protein due to the covalent attachment to the protein molecules of hydrophilic MD glucose polymer side chains, and modification of the net charge of the protein, contributing to greater repulsion between the protein molecules. The increase in the net negative charge of the protein on conjugation with carbohydrate may be attributed to the consumption of charged amino acids, such as the basic amino acid lysine during the Maillard reaction (Ames, 1998; Brands & van Boekel, 2002; Lertittikul *et al.*, 2007; Wang & Zhong, 2014). Interestingly, it was noted that, at similar extents of conjugation, the conjugated sodium caseinate-MD10 had higher protein solubility (~50-80% increase) across the pH range 4.0-4.5 than the conjugated sodium caseinate-MD4.

Similar results were reported by Shepherd *et al.* (2000) and Oliver *et al.* (2006b) on conjugation of sodium caseinate with MD under dry heating conditions, leading to increases in protein solubility, particularly at pH 4.0-4.6, which was again attributed to increased steric repulsion between conjugated protein molecules. Grigorovich *et al.* (2012) reported that sodium caseinate conjugated with MD with DE values of 2 or 10, under dry heating conditions at an initial pH of 7, at 60°C and 79% RH for 72 h, had improved solubility (~10-80% increase) across the pH range 3.5–5.0, compared with sodium caseinate alone. The authors reported that the

improvement in protein solubility of the sodium caseinate-MD conjugate solutions was determined mainly by the molar ratio of the protein:carbohydrate and the DE value of the MD used for conjugation. Similar to the study of O'Regan and Mulvihill (2009b), the most pronounced increase in solubility was achieved using MD with the higher DE value (i.e., DE 10).

The majority of studies on conjugation of casein-based ingredients have been completed using dry heating approaches (Corzo-Martínez *et al.* 2010a,b; Corzo-Martínez *et al.*, 2012b; Markman & Livney, 2012; Morris *et al.*, 2004) while a limited number of studies have been completed using wet heating approaches (e.g., Cardoso *et al.*, 2011). Darewicz *et al.* (1998) showed that a  $\beta$ -casein-glucose solution, heated at an initial pH of 7.4, at 37°C for 24 h under wet heating conditions to achieve conjugation, had a ~5-30% improvement in solubility across the pH range 2.0-8.0, compared to the unheated  $\beta$ -casein control, with the greatest improvement in solubility occurring around the isoelectric point of the protein (~pH 4.6). This improvement in solubility of  $\beta$ -casein was attributed to the covalent attachment of glucosyl residues to  $\beta$ -casein, resulting in an increased hydrophilicity and steric hindrance of the  $\beta$ -casein-glucose conjugates, compared with the unheated mixture. Similar results were also described by Groubet *et al.* (1999) who prepared conjugated  $\beta$ -casein with either arabinose, lactose or ribose (molar ratio 1:100, protein:carbohydrate) by heating at 60°C for 3 d in an anaerobic, aqueous environment and reported that all  $\beta$ -casein-sugar conjugates had increased solubility at pH 4.5–6.0.

However, it should be noted that conjugation of milk proteins with carbohydrates does not always result in increased protein solubility as the type and extent of modification of the functional properties are very dependent on the nature of

the reactants, reaction conditions and the pathways followed by the Maillard reaction (Hiller & Lorenzen, 2010). Corzo-Martínez *et al.* (2012b) reported that dry heating of sodium caseinate and galactose, at an initial pH of 7.0, at 50-60°C, 67% RH, for 4 and 72 h, resulted in a 20% reduction in the solubility of the protein at pH 7.0, compared to the unheated sodium caseinate control. The authors attributed the decreased protein solubility on conjugation to an increase in the surface hydrophobicity of the protein on heating. However, at pH 5.0, Corzo-Martínez *et al.* (2012b) reported that conjugated sodium caseinate-galactose displayed an increase of ~10% in solubility, compared to the unheated and dry heated sodium caseinate controls due to the shift in the isoelectric point of the conjugated protein to a lower pH as a result of a moderate increase in its net negative charge following conjugation.

Heat treatment of proteins can lead to the formation of reactive intermediates (e.g., methylglyoxal or dehydroalanine), which can then react with the  $\epsilon$ -amino group of lysine, resulting in the formation of protein crosslinks, leading to modification of functional properties and loss of nutritional value (Calabrese *et al.*, 2009; Le *et al.*, 2013; Pellegrino *et al.*, 1999). In particular, the development of lysinoalanine (LAL) in protein solutions during heat treatment has been reported to be responsible for protein crosslinking (Gerrard, 2002). Mulcahy *et al.* (2016b) reported that a WPI solution heated for 8 h at 90°C had a higher level of LAL (179 mg 100 g<sup>-1</sup> protein) than the level of LAL in a solution of WPI conjugated with MD6 (58.8 mg 100 g<sup>-1</sup> protein) under the same heating conditions, as the  $\epsilon$ -amino groups of lysine are also consumed by the covalent attachment of carbohydrate to protein during the Maillard reaction, competition for the  $\epsilon$ -amino reaction sites is likely to be responsible for the

lower levels of LAL found in the conjugated protein-carbohydrate solution, compared to protein solution heated alone (Mulcahy *et al.*, 2016a).

The dry heating approach has also been used extensively to conjugate whey proteins with carbohydrates as it is claimed to result in less heat-induced conformational changes to the whey protein molecules (Oliver *et al.*, 2006b; Zhu *et al.*, 2008) as lower temperatures are typically used (Li *et al.*, 2005) than with wet heating. Wang and Ismail (2012) demonstrated that WPI conjugated with dextran by dry heating at 60°C and 49% RH, for 96 h, had enhanced protein solubility (85.7 and 89.0% increase) at pH 4.5 and 5.5, respectively, when they were subsequently heated to 80°C for 30 min, compared to the respective WPI control. The authors reported that the enhanced solubility of WPI on conjugation with dextran was attributed to suppressed intermolecular protein-protein interactions, along with structural/physicochemical changes to the protein, including a shift in the isoelectric point of the protein to a more acidic pH, reduction in the surface hydrophobicity of the whey protein molecules, and increased resistance to thermal denaturation, resulting in a reduced exposure of free sulfhydryl groups after conjugation of the protein with dextran.

A further study by Wang *et al.* (2013) characterised the structural changes in whey protein molecules conjugated with dextran (at 60°C and 49% RH for 96 h) using surface-enhanced Raman spectroscopy. The authors reported that the Raman spectra of the conjugated WPI-dextran samples had an additional peak at 983 cm<sup>-1</sup>, which they attributed to the formation of a Schiff base, and this was accompanied by ionisation of carboxyl groups, contributing to higher net negative charge and re-organisation of the sulphide linkages. These conformational changes in the whey protein molecules

imparted structural rigidity to the conjugated WPI-dextran system, which in turn increased protein solubility on thermal treatment (75°C for 30 min) over a wide pH range (3.4-7.0), compared to previously unheated WPI. Wang *et al.* (2013) also reported that the  $\beta$ -sheet configuration of the whey protein molecules in the conjugated WPI-dextran had increased band intensity in the Raman spectra, compared to that of the unheated WPI control. Wang *et al.* (2013) and Damodaran (2008) reported that the  $\beta$ -sheet configuration is more thermally stable than the  $\alpha$ -helix and other disordered structure configurations in whey protein molecules, thus an increase in the  $\beta$ -sheet configuration may explain the improvements in the thermal stability at pH 4.5 and 5.5 of the conjugated WPI-dextran.

Martinez-Alvarenga *et al.* (2014) studied the effect of temperature, time, water activity and molar ratio of reactants on the functional properties of WPI conjugated with MD ( $M_w$  of 1.7 kDa); the WPI-MD with the lowest extent of conjugation (dry heated at 50°C, 50% RH, for 24 h in the molar ratio of 1:1 protein:carbohydrate) had an increase in protein solubility of just 3% at pH 5.0, compared to the unheated control, due to the covalent attachment of the hydrophilic MD to the protein molecules. The authors reported that WPI-MD with the greatest extent of conjugation (achieved by heating at 50-60°C, 80% RH, for 48 h in the molar ratio of 1:1 or 1:2 protein:carbohydrate) had a shift in the isoelectric point, from pH 5.0 for the unheated control, to pH 4.0-4.5, due to the consumption of positively charged lysine residues during conjugation.

Jiménez-Castaño *et al.* (2007) conjugated individual whey protein fractions ( $\beta$ -lg;  $\alpha$ -lac; BSA) with dextran ( $M_w$  of 10 or 20 kDa) by dry heating at an initial pH of 7.0, at 60°C and 44% RH for 24-72 h. The authors reported that the extent of



conjugation decreased in the following order; BSA> $\beta$ -lg> $\alpha$ -lac and demonstrated that conjugation of  $\beta$ -lg with dextran (20 kDa), for either 36 or 60 h, improved its solubility by ~40% at pH 5.0. However, the solubility of the  $\beta$ -lg-dextran conjugate was ~20-30% lower at pH 4.0, compared to the unheated or heated  $\beta$ -lg control samples, which may be attributed to the consumption of positively charged amino groups (i.e., lysine) causing a shift in the isoelectric point to a more acidic pH. In contrast, Chevalier *et al.* (2001) reported that  $\beta$ -lg conjugated with galactose, glucose, lactose or rhamnose, by wet heating at an initial pH of 6.5, at 60°C for 72 h, had increased solubility of ~25% at pH 4.5, compared to the respective heated  $\beta$ -lg control due to changes in the conformation and hydrophobicity of the protein molecules. Jiménez-Castaño *et al.* (2007) also reported that  $\alpha$ -lac-dextran conjugates exhibited a higher solubility (~5-50% increase), compared to the unheated control, in the pH range 3.0–5.0, with the greatest increase in solubility occurring at pH 4.0; similar trends were reported for BSA–dextran conjugates which had higher solubility around the isoelectric point (pH 4.7–4.9) than the unheated control.

A limited number of studies have reported on modification of the functional properties of whey proteins conjugated with carbohydrates using wet heating conditions. The likely reason for this is that heating of whey protein in an aqueous environment at  $\geq 70^\circ\text{C}$  can result in denaturation and aggregation, which have been reported to reduce whey protein solubility (Liu *et al.*, 2012; Pelegrine & Gasparetto, 2005; Zhu *et al.*, 2010). However, Jiang and Brodkorb (2012), Lillard *et al.* (2009) and Liu and Zhong (2015) have investigated the use of high temperatures (95-130°C) to induce conjugation of whey proteins or isolated whey protein fractions with carbohydrates, and have reported improvements in the antioxidant activity,

emulsification properties and heat stability, respectively, of whey protein-carbohydrate conjugates.

Some alternative approaches to achieving conjugation have also shown promise in increasing protein solubility; for example, Sun *et al.* (2011a) reported that WPI-dextran conjugates, prepared by application of a pulsed electric field (15 and 30 kV cm<sup>-1</sup>, flow rate ~30 ml min<sup>-1</sup>) at an initial pH of 10 and at 30°C for 7.35 ms, had higher solubility (10-30% increase) at pH 4.0-6.0 than the control WPI solution treated with pulsed electric field.

#### *1.11.2 Heat Stability*

Glansdorff *et al.* (1973) defined thermal or heat stability as the ability of a substance to resist irreversible change in its chemical or physical structure, often by resisting polymerisation, under defined conditions (i.e., temperature, pH and ionic strength). Protein-carbohydrate conjugation *via* the Maillard reaction has been shown to be an effective approach to improving the thermal stability of milk proteins. Zhu *et al.* (2010) conjugated WPI with dextran (M<sub>w</sub> 440 kDa) by heating a solution of 10% WPI and 30% dextran, at an initial pH of 6.5 at 60°C for 48 h. The authors measured the thermal stability of the conjugated WPI-dextran solution (0.1%, w/v, protein) by heating at 80°C for 30 min and subsequently measuring the development of turbidity in the solutions (i.e., with increasing development of turbidity there was a higher absorbance at 500 nm; Abs<sub>500</sub>), across the pH range 3.0-7.5. The absorbance of the conjugated WPI-dextran solution did not change on heating; however, there was a ~10 fold increase in Abs<sub>500</sub> of the WPI solution that was heated at 80°C for 30 min in the pH range 4.5-5.5, which was attributed to the formation of large protein aggregates

that scattered light. The authors reported that the unheated WPI had a typical differential scanning calorimetry (DSC) denaturation profile, with an endothermic peak at  $\sim 74^{\circ}\text{C}$  attributed to the denaturation of  $\beta$ -lg, and a shoulder at  $\sim 66^{\circ}\text{C}$ , attributed to the denaturation of  $\alpha$ -lac; however, the conjugated WPI-dextran solution had a flat line profile suggesting that whey protein in the WPI had less secondary structure, due to the covalent attachment of the dextran which contributed to a higher denaturation temperature and improvements in thermal stability. Similar DSC profiles were reported by Hattori *et al.* (1994), Liu and Zhong (2013) and Wang and Ismail (2012) who showed that the denaturation temperature of whey protein-carbohydrate conjugates was higher than that of the corresponding unconjugated whey proteins.

Chevalier *et al.* (2001) reported that  $\beta$ -lg conjugated with either ribose, arabinose, glucose, galactose, lactose or rhamnose, at pH 6.5 and  $60^{\circ}\text{C}$  for 72 h in an aqueous environment (0.4% protein, 0.4% carbohydrate), exhibited greater thermal stability at pH 5.0, when heated at  $70\text{--}90^{\circ}\text{C}$  for up to 1 h, than unheated and heated  $\beta$ -lg controls (i.e., without added carbohydrate). The improvement in thermal stability of the solution (0.2%, w/v, protein), as measured by the concentration of protein in the supernatant of the heated solutions after centrifugation (15 min at 15,000 g), was dependent on the carbohydrate as follows; ribose > arabinose > rhamnose > glucose = galactose > lactose. However, the choice of carbohydrate used in conjugation is known to alter the extent of protein-carbohydrate conjugation, making it difficult to distinguish if the changes in the functional properties were due, directly, to compositional/structural differences between the carbohydrates or, indirectly, to their differing effects on the extent of conjugation (Chen *et al.*, 2013a; Li *et al.*, 2009; Mulcahy *et al.*, 2016a, Chapter 2; ter Haar *et al.*, 2011).

Liu and Zhong (2013) conjugated WPI with either glucose, lactose or MD ( $M_w$  1 kDa) by dry heating at an initial pH of 7.0 at 80°C and 80% RH for 2 h, in a mass ratio of 1:1, and evaluated heat stability by reconstituting samples to 7%, w/v, protein, adding 0-150 mM NaCl or CaCl<sub>2</sub>, adjusting the solutions to pH between 3.0-7.0, and heating for 2 min at 88°C, simulating a hot-fill beverage process (Etzel, 2004). The authors assessed the thermal stability by visual observation of turbidity development after heating; the solutions prepared from the conjugated WPI-MD and WPI-lactose remained transparent under all conditions tested, while the unheated and dry heated WPI controls with added salt became turbid on heating at pH 6.0. Liu and Zhong (2013) reported that the conjugated WPI-MD had a higher denaturation temperature and a more negative net charge across the pH range 2.0-7.0 than the unheated WPI control, which may have contributed to the increased thermal stability of the former. However, they concluded that the main mechanism for the improvement in thermal stability was the increased steric hindrance between the protein molecules provided by the attachment of carbohydrate to the protein molecules.

Several authors reported that improvements in heat stability of whey protein-carbohydrate conjugates can be related to the number and chain length of the carbohydrates attached to the whey protein molecules, along with the location at which they are attached on the protein molecules; the attachment of higher  $M_w$  carbohydrates has been shown to have a greater impact on improving the thermo-stability of whey proteins, due to increased steric repulsion, compared to conjugation with monosaccharides (Aoki *et al.*, 1999; Corzo-Martinez *et al.*, 2012b, c; Morris, *et al.*, 2004; Mulcahy *et al.*, 2016a, Chapter 3; Tuinier *et al.*, 2002; Wong *et al.*, 2011; Wooster & Augustin, 2006).

WPHs have been reported to have impaired functional properties compared to their intact counterparts and have been shown to be more susceptible to destabilisation when heated, due to the exposure of buried hydrophobic residues and/or release of specific peptides that promote peptide-peptide and peptide-protein aggregation (Adjonu *et al.*, 2013; Creusot & Gruppen, 2007). Mulcahy *et al.* (2016b) reported that WPH (DH 9.3%) conjugated with MD (DE 17) under wet heating conditions at an initial pH of 8.2 and 90°C for 8 h, had superior thermal stability to further heating at 85°C for 10 min with 40 mM NaCl added, compared to those of the unheated or heated WPH control solutions. The unheated or heated WPH control solutions precipitated and phase separated on heating at 85°C for 10 min due to the formation of large protein aggregates (~10-50 µm), whereas, the conjugated WPH-MD solution (i.e., previously heated for 8 h at 90°C at an initial of pH 8.2) that was further heated with 40 mM added NaCl, remained stable and the protein aggregates present remained small (<~1 µm).

The conditions used during the Maillard reaction impact the thermal stability of the resulting conjugates; Wang and Zhong (2014) dry heated WPI-MD in the mass ratio 1:1, at 80°C and 65% RH for 4 h, at different pH (i.e., pH 4.0, 5.0, 6.0 and 7.0). The solutions prepared from the conjugated WPI-MD at pH 6.0 (5% protein, and 0-150 mM added NaCl) that was subsequently heated at 138°C for 1 min (to simulate UHT treatment), had improved thermal stability (i.e., remained transparent as evaluated by the visual assessment of turbidity) compared to the solution prepared from the WPI-MD conjugated at pH 4.0. The improvement in thermal stability was attributed to the greater extent of covalent attachment of MD molecules to the whey protein molecules at pH 6.0, resulting in reduced protein-protein interactions, lower

surface hydrophobicity of the protein, a shift in the isoelectric point (from 4.63 to 4.07) and a higher protein denaturation temperature compared to the WPI-MD conjugate prepared at pH 4.0.

Protein-carbohydrate conjugates produced by alternative methods, such as sonication, have also been shown to have improved functionality; Perusko *et al.* (2015) conjugated a WPI-arabinose-polyethylene glycol (PEG) solution at an initial pH of 8.0, at ~5-10°C by sonication (20 kHz frequency) for 60 min. The authors reported that the conjugated WPI-arabinose-PEG solution had a greater extent of conjugation (10% increase), due to the presence of PEG facilitating macromolecular crowding, compared to the sonicated solution of WPI-arabinose without PEG. The conjugated WPI-arabinose-PEG solution had higher protein solubility (~10-40% increase) when the solution was heated at 80-100°C for 15 min compared to the sonicated WPI control solution and the conjugated WPI-arabinose solution without PEG. The authors attributed this increase in thermal stability of the conjugated WPI-arabinose-PEG solution to attachment of the arabinose units to the whey protein molecules, which interfere with protein aggregation due to steric hindrance limiting protein-protein interactions.

Protein-carbohydrate conjugation also enhances the functional properties of WPC; Liu and Zhong (2014) prepared a defatted WPC (34% protein) by adjusting a WPC solution to pH 4.0, centrifuging, and spray drying the resulting supernatant. The resulting defatted WPC was conjugated by heating under dry conditions, at 130°C for either 20 or 30 min, or 60°C for either 24 or 48 h, at 79% RH, which resulted in the whey proteins conjugating with the innate lactose (68.8%) present in the ingredient. The authors assessed the thermal stability of solutions (4%, w/v, protein) prepared

from the conjugated WPC by adjusting the pH of the solution to 3.0-7.0 and heating the solutions at 88°C for 2 min or at 138°C for 1 min. The WPC conjugated at 130°C for 30 min remained transparent once further heated at 138°C for 1 min with 150 mM added NaCl; however, the authors did not suggest a mechanism for the apparent improvement in thermal stability.

Conjugation has also been shown to be beneficial in producing heat stable whey protein nanofibrils; Liu and Zhong (2013) produced protein nanofibrils (pH 2.0, heated at 85°C for 24 h) from solutions of WPI and lactose which had previously been conjugated under dry heating conditions (80°C and 70% RH for 2 h). The nanofibrils prepared from the conjugated WPI-lactose were highly dispersible and remained transparent after heating (88°C for 2 min or 138°C for 1 min) in the pH range 4.0–7.0, even with up to 150 mM NaCl added, compared to the nanofibrils formed from a WPI solution, which became turbid under all heating conditions tested. The greater thermal stability of the nanofibrils produced from the conjugated protein was attributed to the lactose on the nanofibril surface providing additional steric hindrance.

### *1.11.3 Emulsification*

There has been considerable growth in the area of modification of emulsification properties of proteins by their conjugation with various carbohydrates through the Maillard reaction (Drapala *et al.*, 2016a,b; de Oliveira *et al.*, 2016; Foegeding & Davis, 2011; Liu *et al.*, 2012; Oliver *et al.*, 2006a). Protein-carbohydrate conjugates consist of two composite blocks, where, in an emulsion system, the more surface-active component (i.e., protein) adsorbs at the oil/water (O/W) interface, while the more hydrophilic component (i.e., carbohydrate) extends into the bulk aqueous

phase of the emulsion; the two components display two distinct, complimentary and synergistic roles in bringing about the action of conjugate-based emulsifiers.

Conjugation of proteins with carbohydrates can improve their emulsion formation properties indirectly by enhancing protein solubility, increasing their effective concentration and mobility in aqueous solution. Carbohydrate moieties covalently attached to protein on conjugation act like a tail, and are effectively towed by the protein as it migrates through the bulk aqueous phase towards the O/W interface, as the carbohydrate generally does not provide a driving force for this migration of the conjugated protein molecules. Despite its more passive role in the formation of emulsions, the carbohydrate component of protein-carbohydrate conjugates generally does not impede the movement of the conjugated protein through the bulk phase, except when the size ratio between the protein and carbohydrate is disproportional. The larger hydrodynamic radius of protein-carbohydrate conjugates, compared to the protein alone, can potentially result in a decreased rate of diffusion in the bulk phase and reduce the rate of adsorption of conjugates at the interface (Ganzevles *et al.*, 2007).

The improved emulsion formation properties of milk protein-carbohydrate conjugates, compared to unconjugated protein, can be also attributed to their strong steric stabilisation properties; as the emulsifier adsorbs at the surface of newly formed oil globules on homogenisation, it prevents their coalescence by means of steric repulsion (Liu *et al.*, 2016). The carbohydrate moiety anchored at the surface of an oil globule by the protein, protrudes into the aqueous phase of the emulsion and prevents coalescence on high impact collisions between individual oil globules during the dynamic homogenisation process (Corzo-Martínez *et al.*, 2011).



Conjugation of milk proteins with carbohydrates generally enhances their emulsion formation and stabilisation properties at high salt concentrations and under acidic conditions, due to improved protein solubility under such environmental conditions. Drapala *et al.* (2016a,b) showed that model infant formula emulsions stabilised by WPH-MD conjugates, produced by a wet heating approach, were resistant to heat-induced bridging flocculation, compared to those stabilised by non-conjugated WPH. The authors reported that the conjugate-stabilised systems showed no changes in viscosity or particle size distribution after a high temperature-short time (HTST) treatment of between 75-100°C for 15 min, in contrast to emulsions stabilised by intact, hydrolysed or pre-heated hydrolysed whey protein. The improved thermal stability of emulsions stabilised by conjugated protein was attributed to the physical restriction of access (by serum phase constituents such as un-adsorbed proteins) to the potentially reactive inner interfacial layer (i.e., protein) by the unreactive outer interfacial layer (i.e., carbohydrate).

Wooster and Augustin (2006) reported that the thickness of the interfacial layer in O/W emulsions stabilised by  $\beta$ -lg-dextran conjugates can be modified by using carbohydrates with different  $M_w$ . Fundamentally, increasing the  $M_w$  of the carbohydrate moiety yields increased thickness of the interfacial layer and confers greater steric stabilisation as a result (Akhtar & Dickinson, 2007). However, factors such as the kinetics of conjugation and the rate of diffusion/adsorption of the conjugate can both be negatively impacted by increasing  $M_w$  of the carbohydrate and need to be considered when using higher  $M_w$  carbohydrates.

Emulsions stabilised by conjugated milk proteins display greater oxidative stability than those stabilised by protein alone, possibly due to the increased thickness

of the interfacial layer and the physical barrier that restricts the access of pro-oxidant species to oxidation-sensitive components such as lipids and lipid-soluble compounds. A significant improvement in the oxidative stability of emulsions containing  $\beta$ -carotene, stabilised by lactoferrin conjugated with dextran, compared to emulsions stabilised by the protein alone, was reported recently by Liu *et al.* (2016), where the anti-oxidative effect was attributed to restriction of physical contact between pro-oxidants and lipids by the thick interfacial layer of the conjugate-stabilised emulsion. Furthermore, it has been shown that certain late-stage Maillard reaction products (e.g., melanoidins) have antioxidant properties when incorporated into O/W emulsions (Markman & Livney 2012; O'Regan & Mulvihill, 2010). Milk protein/peptide-carbohydrate conjugate-based emulsifiers also offer potential for applications in emulsion-based delivery systems, where their interfacial functionality can facilitate controlled release of sensitive bio-actives (e.g., vitamins) in the small intestine, avoiding acid-mediated emulsion destabilisation and loss of the encapsulated material in the stomach (Lesmes & McClements, 2012).

#### *1.11.4 Foaming*

Stability of foams is inherently lower than that of emulsions, due to the greater tendency to separation of the two phases, owing to the larger diameter and lower density of the dispersed phase particles (i.e., air bubbles and oil globules, respectively) in foams than in emulsion systems. In addition, solubility of the dispersed phase in the continuous phase and macroscopic processes such as liquid drainage, Ostwald ripening (i.e., disproportionation) and bubble coalescence also contribute to foam instability. Changes to structure/conformation of proteins, resulting from their

conjugation with carbohydrates, generally contribute to increased protein solubility, higher protein mobility and, effectively, faster adsorption at air/water (A/W) interfaces. Improvement in foam capacity of BSA conjugated with glucose in a wet heating process (45°C for 2 h), compared to BSA conjugated with mannose or unconjugated BSA, was reported by Jian *et al.* (2016). In this study, conjugation resulted in changes in protein conformation, yielding a more flexible and loosened structure which, effectively, increased the rate of protein adsorption at the A/W interface. Similar findings were reported for foams stabilised by  $\beta$ -lg-glucose conjugates (dry heating; 50°C at 65% RH for 96 h) (Báez *et al.*, 2013), where improved foam capacity, compared to using unconjugated  $\beta$ -lg, was explained by heat-induced conformational changes in the structure of the whey protein molecules, conferring more open and flexible structures, thus allowing more rapid formation of the interfacial layer.

Other approaches directed at improving foam stability involve increasing the thickness and elasticity of the interfacial film by increasing the size of its building blocks (i.e., controlled protein aggregation) (Báez *et al.*, 2013; Dombrowski *et al.*, 2016; Foegeding *et al.*, 2002) or by conformational changes to the protein structure (i.e., partial unfolding of globular protein) (Dissanayake & Vasiljevic, 2009; Dombrowski *et al.*, 2016; Morales *et al.*, 2015). When using protein/peptide-carbohydrate conjugates to stabilise foams, the thickness of the interfacial layer and therefore, effectiveness of steric stabilisation, can be controlled using carbohydrates with different  $M_w$  (Wooster & Augustin, 2006). Hiller and Lorenzen (2010) reported increased stability of foams prepared with a range of protein (WPI, sodium caseinate and lactose-hydrolysed skim milk) and carbohydrate (glucose, lactose, pectin and

dextran) conjugates (produced by dry heating at 70°C and 65% RH for up to 240 h) due to formation of thick and viscoelastic interfacial films that prevented disproportionation of gas bubbles. Increasing the thickness of the interfacial film can improve its rheological properties in addition to providing an effective steric barrier with good dilatational properties (Dombrowski *et al.*, 2016). Similarly, Kim *et al.* (2005) reported that denaturation and unfolding of  $\beta$ -lg resulted in increased shear elasticity and viscosity of the interfacial layer due to increased flexibility of the partially-denatured globular protein. Cai and Ikeda (2016) reported increased resistance against surfactant-induced displacement of protein from the A/W interface in foams stabilised with WPI-gellan conjugates prepared by dry heating at 80°C and 79% RH for 2 h, compared to systems containing unconjugated WPI and the surfactant Tween 20. The authors attributed the greater resistance to displacement of protein in the conjugate-based foam system to the ability of the gellan moiety, covalently attached to the whey protein molecules, to form a carbohydrate network at the interface, effectively immobilising the conjugate-covered interface.

Conjugation of protein with carbohydrates alters the hydrophobic-hydrophilic balance of protein and conformational changes to the protein structure caused by conjugation increase its surface hydrophobicity, generally resulting in improved emulsion formation properties of conjugated proteins (see Section 1.11.3). On the other hand, hydrophilicity of the resulting ingredient is increased by the attachment of the hydrophilic carbohydrate moieties (see Section 1.11.1). Conversely, greater hydrophilicity can yield better foam stability due to improved water holding capacity by the conjugate located at the interfacial layer, and effectively restrict liquid drainage in the foam (Báez *et al.*, 2013). The hydrophilic nature of the carbohydrate anchored

at the A/W interface by the protein, viscoelastic properties of the interface and higher viscosity for conjugated protein-carbohydrate systems (WPI, SMP, sodium caseinate, glucose, lactose, pectin, dextran), compared to native protein, have been shown by Hiller and Lorenzen (2010) to be the main factors responsible for increased foam stability. In contrast, other authors have claimed that the increased hydrophilicity of BSA resulting from its conjugation with glucose or mannose decreased foam stability (Jian *et al.*, 2016). Jiang and Zhao (2011) elucidated that a shift in the amphipathic nature of casein towards more hydrophilic behaviour, upon conjugation with glucosamine, reduced ability of foam to retain the incorporated air. It is important to consider that both of these, apparently contradictory, findings can hold true, with the precise impact of conjugation being very much dependent on differences in protein structure (globular, ordered, unordered etc.), nature of the carbohydrate (chain length, charge etc.) and conditions employed for conjugation and foam formation.

#### *1.11.4 Gelation and Textural Properties*

Whey protein gels are three-dimensional, self-supporting, networks, within which the aqueous solution and any dispersed elements (e.g., fat) are entrapped. Gelation of whey proteins involves a controlled increase in protein-protein interactions, while carefully maintaining a balance with protein-solvent interactions (Brodkorb *et al.*, 2016). During gelation, the number and combined strength of protein-protein interactions (e.g., disulphide, hydrophobic and electrostatic interactions) determine the mechanical and rheological properties of the resultant gel network. High whey protein content ingredients (i.e., WPC and WPI) are commonly used in food applications which require gelation of the protein for the expression of

functionality (e.g., recombined meat products, desserts, puddings, mousses). Many compositional and environmental factors affect the formation and rheological properties of whey protein gels, including protein concentration, pre-denaturation and aggregation of protein, salts, temperature and pH (Foegeding *et al.*, 1995; Langton & Hermansson, 1992; Mulvihill & Kinsella, 1987).

It is known for over 20 years that heating solutions of globular milk proteins (e.g., lysozyme and BSA) and reducing sugars (e.g., lactose, ribose and xylose), at temperatures of 90-121°C, results in the formation of gels with higher firmness and elasticity than gels made using the proteins alone in solution (Armstrong *et al.*, 1994; Easa *et al.*, 1996). The increased strength of these protein-carbohydrate gels is due to Maillard reaction-mediated reduction in pH and cross-linking of the protein molecules (e.g., *via* lysinoalanine). The gel strength (but also, undesirably, colour development) increases with decreasing  $M_w$  of the sugars (Hill *et al.*, 1992), while the pH required to achieve gelation decreases with increasing sugar concentration and reactivity. In combination, these effects of sugar incorporation on gelation properties of globular protein on heating, means that it is possible to reduce the amount of protein required for gel formation (Azhar, 1996; Oliver *et al.*, 2006b).

More recent work has focused on studying the gelation properties of milk proteins (especially whey proteins) conjugated with higher  $M_w$  carbohydrates under dry heating conditions, due to the challenges associated with denaturation and aggregation of whey proteins under wet heating conditions (Gauthier *et al.*, 2001; Morgan *et al.*, 1999a). Conjugation of whey proteins in WPI with dextran has been shown to influence the rheological properties of heat-induced gels made therefrom (Spotti *et al.*, 2013a,b; 2014a,b; Sun *et al.*, 2011b). Conjugation of WPI with dextran

of  $M_w$  6, 40 and 70 kDa, under dry heating conditions at 60°C for 2-9 d at 63% RH was shown to result in whey protein-conjugate gels with lower fracture stress and Young's Modulus as measured by uniaxial compression testing (Spotti *et al.*, 2013a; 2013b) and lower gel firmness (i.e., storage modulus) as measured by dynamic low amplitude oscillatory shear rheology (Spotti *et al.*, 2014a; 2014b), compared with WPI alone or unconjugated WPI-dextran mixtures. Similar results were reported by Sun *et al.* (2011b) for WPI conjugated with dextran (average  $M_w$  150 kDa) at 60°C for 7 d at 79% RH.

The lower strength of heat-set WPI-based gels made from whey protein conjugated with dextran, compared with unconjugated whey protein or mixtures of whey protein and carbohydrates is attributed to several factors, with the relative contribution of the individual factors dependent on the system composition and conditions of conjugation. Under the heating conditions typically required to achieve conjugation (see Section 1.10), denaturation and aggregation of whey proteins can occur, serving to alter exposure and reactivity of functional groups (e.g., free sulphydryl and hydrophobic groups) and the surface charge of protein molecules, all of which influence protein-protein and protein-water interactions (Brodkorb *et al.*, 2016). Covalent attachment of the carbohydrate molecules also increases the hydrophilicity and steric barrier properties of the conjugated proteins, both of which result in decreased protein-protein interactions and increased protein-water interactions.

#### *1.11.5 Enrichment and Purification of Conjugates*

The improvements in functionality, as outlined above, arising from conjugation of milk proteins/peptides with carbohydrates, are normally associated with relatively low levels of conjugated protein (relative to the total protein concentration of the systems). Thus, it is desirable to enrich the protein-carbohydrate conjugates from the reaction mixtures in which they are produced in order to remove unreacted carbohydrate, unreacted protein and possibly soluble Maillard reaction products, while increasing conjugated protein concentration. Such processes need to be food-grade, efficient, economical and have acceptable yield-enrichment, as opposed to purification, of the protein-carbohydrate conjugate is normally sufficient.

Only limited reports have been published to date on the enrichment/purification of milk protein-carbohydrate conjugates, and the studies that have been reported (Bund *et al.*, 2012; Etzel & Bund, 2011) are very much informed by approaches used in the pharmaceutical industry for purification of various therapeutic proteins conjugated with polyethylene glycol (i.e., PEGylated proteins), with separation being achieved largely based on differences in hydrophobicity (i.e., using hydrophobic interaction chromatography) (Mayolo-Deloisa *et al.*, 2016) and charge density (Abe *et al.*, 2010) between conjugated and unconjugated proteins.

An initial study by Etzel and Bund (2011) involved laboratory-scale, analytical separation and enrichment of whey protein-dextran conjugates from mixtures of unreacted dextran and whey protein using cation exchange column chromatography with traditional chromatographic beads or porous polymethacrylate monolithic media and sodium lactate/sodium chloride-containing elution buffers. Using such an approach, unreacted dextran eluted first, followed by the conjugated protein and



finally the unreacted protein; a portion of the unreacted whey protein was isoelectrically precipitated from the feed stream at pH 5.0, before chromatographic separation. The monolith media resulted in a similar dynamic binding capacity as the traditional beaded support (4-6 g/L) but with 42-fold higher mass productivity and 48-fold higher flow rate, while yielding a conjugate-enriched stream with lower purity. The use of cation exchange chromatography, as originally proposed by Etzel and Bund (2011) has been successfully scaled up to a preparative scale (i.e., 160 fold up-scaling from 5 mL to 800 mL columns) by Bund *et al.* (2012). On scale up, the upfront partial removal of unreacted whey protein by isoelectric precipitation was shown to be effective in reducing the buffer volumes, purification time and the number of chromatography cycles required for purification of the conjugates. The yield of conjugated protein was ~18% on a protein basis, with the losses mainly associated with incomplete conversion of unconjugated to conjugated whey protein during the conjugate production process.

Opportunities for increasing this conversion rate should be evaluated in future studies, with integration of conjugation and fractionation steps to reintroduce unreacted dextran and protein or the use of on-column conjugation having being suggested by Fee and Van Alstine (2006) and Bund *et al.* (2012). In addition, progressively increasing the salt concentration during elution for the enrichment/purification of milk protein-carbohydrate conjugates would be expected to facilitate separation of conjugates based on differences in their degree of glycosylation, as is the case with PEGylated lysozyme and BSA (Abe *et al.*, 2010).

## 1.12 References

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## **Objectives**

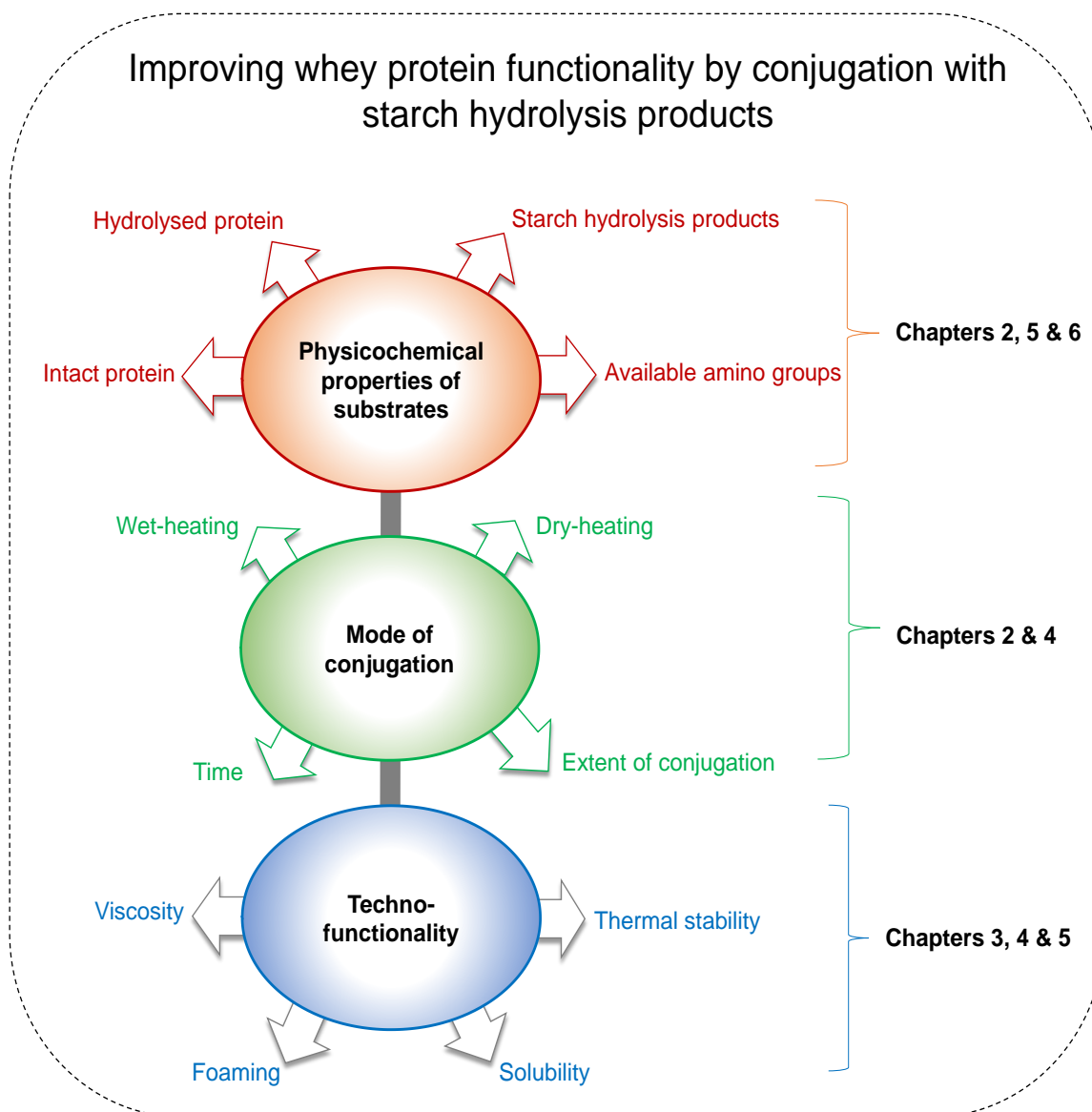
The proteins of milk are considered its most valued constituents from both a techno-functional and nutritional perspective and many milk protein-based ingredients have been developed over the years, which harness the unique functional properties of milk proteins. A rapidly growing application for whey protein ingredients is protein-based beverages (Augustin & Udabage, 2007; Dairymark, 2012; Smithers, 2008); for example, the volume of sports nutritional beverages, most of which are whey protein based, increased from 7563 to 9679 million litres globally between 2004 and 2009, which translates to an average annual increase of ~5% (Cochrane et al., 2012).

Several unit operations applied during process of whey proteins, including heat-treatment and hydrolysis, may result in impairment of their functional attributes (Creusot & Gruppen, 2007; LaClair & Etzel, 2010). Arising from thermal treatment required to extend shelf-life together with the formulation complexities associated with nutritional beverages, challenges, such as turbidity development, protein coagulation, sedimentation and possibly gelation, are often experienced on incorporation of WPI ingredients in nutritional beverages (LaClair & Etzel, 2010). Hydrolysis of whey proteins may also increase their susceptibility to destabilisation when heated, due to the exposure of buried hydrophobic amino acids and/or the release of specific peptides that promote peptide-peptide and peptide-protein aggregation. However, consumer acceptance of nutritional beverages is based on clarity of these beverage solutions, with consumers rating clear beverages as having superior sensory and ‘thirst-quenching’ attributes compared with opaque beverages (Beucler et al., 2005).

As exemplified in the preceding literature review, milk protein functionality continues to be an active area of research with Maillard-induced conjugation of milk proteins/peptides offering considerable potential in the development of milk protein-based ingredients with enhanced heat stability, solubility, emulsification and foaming properties. Data obtained from Scopus (2016) indicates that whey protein-carbohydrate conjugation is a growing field of interest, with the number of academic research publication increasing steadily per year; however, the majority of the published literature has focused on dry heating approaches to induce conjugation of whey proteins (Akhtar & Dickinson, 2007; Lillard et al., 2009; Liu & Zhong, 2012; Martinez-Alvarenga et al., 2014; Neirynck, 2004; Spotti et al., 2014; Wooster & Augustin, 2007; Xu et al., 2012) with much less published literature on the use of wet heating approaches to induce conjugation (Böttger et al., 2013; Jiang & Brodkorb, 2012; Mulcahy et al., 2016a, b; Perusko et al., 2015; Zhu et al., 2008).

The main objective of the research element in this thesis was to develop and characterise whey protein-carbohydrate conjugate ingredients with enhanced physicochemical functionalities for application in nutritional beverages. The work presented was performed with a view to maximising the alteration of physicochemical/functional properties arising from conjugation during the early stages of the Maillard reaction, while minimising the development of advanced Maillard products. The associations between the different research themes are illustrated in Fig. 1.19.

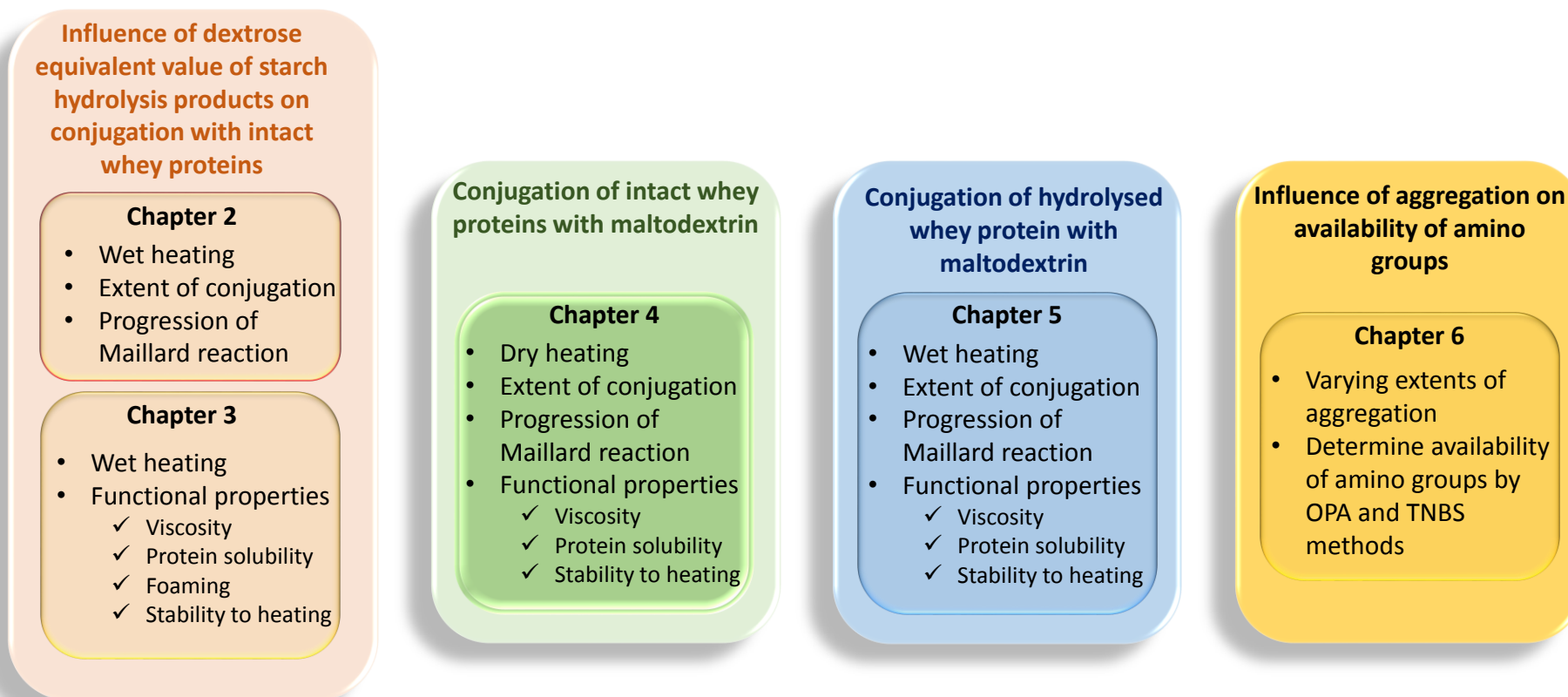




**Fig. 1.19.** Diagrammatic outline of the associations between the core themes investigated in this thesis

A schematic representation of the experimental phases of the thesis are presented in Fig. 1.20. The specific aims of the research are as follows:

- To undertake a systematic evaluation of the influence of starch hydrolysis product chain-length on the rate and extent of conjugation to whey proteins in a heated aqueous environment
- To optimize the level of conjugation while limiting the associated progress of the Maillard reaction to the advanced stages
- To characterise the physicochemical and functional properties of conjugated whey protein-carbohydrate systems prepared using starch hydrolysis products with different dextrose equivalent values
- To investigate the influence of dry heating on the extent of conjugation of whey proteins with maltodextrin and to determine the alteration of physicochemical/functional properties arising from conjugation
- To assess if the physical state of the whey protein (i.e., intact or hydrolysed) affected the extent of conjugation in a heated aqueous environment and to characterise the functional properties of the resulting conjugated systems
- To determine if heat- and salt-induced aggregation of whey proteins impacted the availability of amino groups as measured by the *o*-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods.



*Fig. 1.20. Schematic representation of the experimental phases of the thesis.*

## Chapter 2

### *Characterisation of heat-induced protein aggregation in whey protein isolate and the influence of aggregation on the availability of amino groups as measured by the o-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods*

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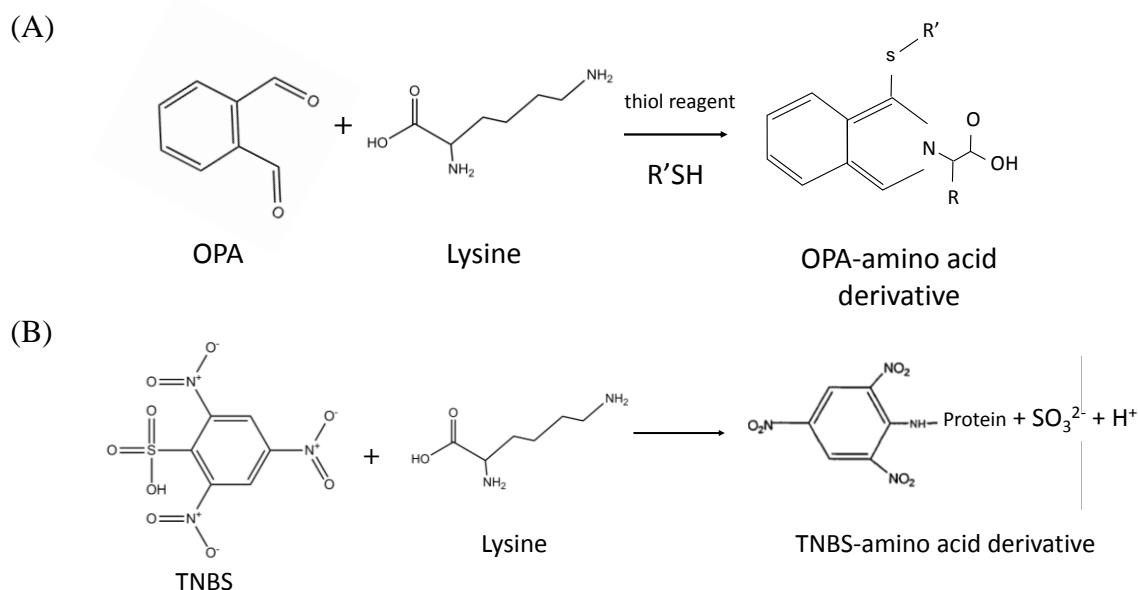
## 2.1 Abstract

Whey protein isolate (WPI) solutions with different levels of aggregated protein were prepared by heating (5% protein, pH 7, 90°C for 30 min) WPI solutions with either 20 mM added NaCl (WPI+NaCl), 5 mM N-ethylmaleimide (WPI+NEM) or 20 mM added NaCl and 5 mM NEM (WPI+NaCl+NEM). Gel electrophoresis demonstrated that the heated WPI and WPI+NaCl solutions had higher levels of aggregated protein, due to increased covalent interactions between proteins, than the heated WPI+NEM and WPI+NaCl+NEM solutions. There were marked differences in the level of amino groups between all heated WPI solutions when measured by the OPA and TNBS methods, with lower levels being measured by the TNBS method than the OPA method. These results demonstrate that the measurement of available amino groups by the OPA method is less impacted than the TNBS method by heat-induced structural changes, arising from disulphide or sulfhydryl-disulphide bond-mediated aggregation of whey protein molecules.

## 2.2 Introduction

It has been recognized for over 100 years that the  $\epsilon$ -amino group of lysine and  $\alpha$ -amino groups of N-terminal amino acids in proteins are largely responsible for the available amino groups (AAG) present in proteins (Skraup & Kaas, 1906). This finding triggered the development of various assays to study accessibility/availability of amino groups in proteins; the *o*-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods have been shown to be both rapid and sensitive for quantifying AAG in protein solutions (Rutherford, 2010a). The OPA reaction consists of two steps where (1) the OPA reagent reacts with the thiol group in the reaction buffer and (2) the OPA-thiol intermediate reacts with the amino group of proteins to form 1-alkylthio-2-alkyl substituted isoindoles which can be quantified by measuring their absorbance at 340 nm (Fig. 2.1A) (Joys & Kim, 1979). The TNBS reagent also reacts specifically with primary amino groups to form coloured trinitrophenyl-amino acid derivatives, which are also measured at an absorbance of 340 nm (Adler-Nissen, 1979; Fig. 2.1B).

Although both methods involve specific reactions between the reagents and the primary amino groups of proteins, several authors have reported discrepancies in measured values for AAG when comparing the OPA and TNBS approaches; these variances have been attributed to instabilities of the OPA and TNBS reagents when reacting with cysteine and the *N*-terminal proline residue of proteins, the presence of insoluble protein material and heat treatment of proteins (Spellman *et al.*, 2003; Rutherford, 2010a).



**Fig. 2.1.** Overview of the reaction of (A) *o*-phthaldialdehyde (OPA) and (B) trinitrobenzenesulfonic acid (TNBS) with amino acids. OPA in the presence of a reducing agent ( $\text{R'SH}$ ) reacts with available amino groups (i.e., terminal amino acids and the  $\epsilon$ -amino group of lysine) to form a UV-detectable isoindole-derivative. TNBS reacts with available amino groups by a nucleophilic aromatic substitution reaction, which converts the free amino groups to a UV-detectable trinitrophenyl-amino acid derivative.

Amino groups can be rendered either nutritionally unavailable or chemically unavailable for further reaction during processing and/or prolonged storage of protein ingredients (Hurrell & Carpenter, 1981); however, the availability of amino groups in the context of this study refers to chemically available amino groups that have not been structurally altered. It is important to determine the availability of amino groups in protein-based ingredients as it can impact on both the bio-availability and techno-functional properties of the proteins (Cattaneo *et al.*, 2009; Rutherfurd, 2010b; Rutherfurd & Moughan, 2005). The  $\epsilon$ -amino group of lysine and  $\alpha$ -amino groups of *N*-terminal amino acids can be rendered either nutritionally unavailable or unavailable for further reaction by several types of heat-induced covalent linkages, including covalent interactions between protein molecules and

carbonyl groups of reducing sugars (e.g., lactose) or by heat-induced aggregation of milk proteins (Jiang, & Brodtkorb, 2012; Hurrell & Carpenter, 1981; Mehta & Deeth, 2015; Mulcahy *et al.*, 2016a).

The thermal stability of whey proteins has been the subject of extensive research and there are many reports in the literature on the denaturation and aggregation of whey proteins under different solution and processing conditions (Anema, 2009; Donovan & Mulvihill, 1987; Marangoni *et al.*, 2000; Oldfield *et al.*, 2005; Ryan *et al.*, 2013; Sağlam *et al.*, 2014). Fitzsimons *et al.* (2007) reported that the denaturation and aggregation processes for whey proteins can be separated into two separate stages where the first stage is denaturation of the native globular whey protein structure and the second stage is rearrangement of the protein structure, resulting in aggregation. The denaturation process is reversible, where dissociation of intramolecular bonds (i.e., non-covalent and, in some cases, disulfide) and partial unfolding of the whey protein molecules takes place (Fitzsimons *et al.*, 2007). At temperatures exceeding 70°C, irreversible aggregation occurs, which results in the formation of aggregates due to sulphhydryl group interaction, disulphide interchanges (Sawyer, 1968; Wijayanti *et al.*, 2014a), along with the participation of non-covalent hydrophobic and/or electrostatic interactions (Hoffmann & van Mil, 1997; Joyce *et al.*, 2016; Verheul *et al.*, 1998).

The molecular size, physicochemical and functional properties of whey protein aggregates are strongly influenced by the ionic strength of the environment, heating conditions, concentration and charge of the proteins, and mechanism of aggregation (Vardhanabhuti *et al.*, 2001). The presence of ions (e.g., Na<sup>+</sup>, Ca<sup>2+</sup>) at sufficient concentrations in whey protein solutions can result in intermolecular



electrostatic shielding of negatively charged proteins and ion-induced structural changes that facilitate hydrophobic interactions between protein molecules resulting in extensive aggregation of whey proteins on heating (Majhi *et al.*, 2006; Schokker *et al.*, 2000; Verheul *et al.*, 1998). Soluble whey protein aggregates are formed at neutral pH, in low ionic strength environments where increased electrostatic repulsion between the protein molecules results in the formation of mainly disulfide bond-mediated aggregates (Ikeda & Morris, 2002; Schmitt *et al.*, 2007; Vardhanabhuti & Foegeding, 1999).

Reaction with the disulphide blocking reagent, *N*-ethylmaleimide (NEM) has been widely reported to limit aggregation of whey proteins by inhibiting some of the disulfide or sulfhydryl-disulfide mediated interactions by nucleophilic attack on the sulfhydryl group of cysteinyl residues of proteins (Croguennec *et al.*, 2003; Smyth *et al.*, 1964). Hoffmann and van Mil (1997) reported that the presence of NEM during heating of  $\beta$ -lg solutions (1-5% protein, pH 7) was found to prevent the formation of  $\beta$ -lg polymers on heating at 65°C for up to 48 h. Kitabatake *et al.* (2001) reported that although disulphide interchanges were limited in a  $\beta$ -lactoglobulin ( $\beta$ -lg) solution (1% protein, pH 7.5) containing NEM (1.0 mM) heated at 80 °C for 1 h, other non-covalent bonding (e.g., hydrophobic interactions) can still contribute to whey protein aggregation, particularly in high ionic strength environments.

Many authors have reported a decrease in available lysine in milk proteins on heating which is related to the severity of the thermal treatment applied (Finot, 1983; Mehta & Deeth, 2015). Solutions of  $\beta$ -lactoglobulin ( $\beta$ -lg) heated at 60°C for 72 h had 5.0% less AAG compared to the unheated control when measured by the OPA method (Chevalier *et al.*, 2001). Cayot and Tainturier (1997) reported that only

5 of the total 15 amino groups were available (as measured by the TNBS method) in a  $\beta$ -lg solution (4.5% protein) at 20°C as the amino groups are buried within the compact spatial structure of the protein molecules. However, the main focus of these studies was on the consumption of AAG as an indicator of the extent of protein-carbohydrate conjugation. Little detailed information appears to be available in the peer-reviewed scientific literature on the influence of aggregation of whey proteins on the availability of amino groups.

The focus of this study was to determine systematically the influence, if any, of heat-induced aggregation of whey proteins on the availability of amino groups as measured by the OPA and TNBS methods. The conditions used in this study were selected to provide varying extents of aggregation of the whey protein in solution; salt was added to the whey protein solutions to promote interactions and aggregation of the whey protein molecules, whereas NEM was added to inhibit the formation of disulfide and sulfhydryl-disulfide-mediated whey protein aggregates. The extent of denaturation and aggregation of whey proteins were characterised in detail and the influence of heat-induced aggregation on the availability of amino groups in whey protein molecules as measured by the OPA and TNBS methods was determined.

## 2.3 Materials and Methods

### 2.3.1 Materials

Whey protein isolate (WPI), BiPro<sup>®</sup>, was obtained from Davisco Foods International (Le Sueur, MN, USA). Protein (89.4%), ash (3.0%), moisture (4.5%), fat (0.4%) and lactose (0.3%) content of the WPI were determined using standard analytical procedures as detailed by Mulcahy *et al.* (2016). Sodium (747 mg 100 g<sup>-1</sup> powder), calcium (70 mg 100 g<sup>-1</sup> powder), phosphorus (58 mg 100 g<sup>-1</sup> powder), potassium (29 mg 100 g<sup>-1</sup> powder) and magnesium (4.7 mg 100 g<sup>-1</sup> powder) content in the WPI were measured using inductively-coupled plasma-mass spectrometry, as described by Herwig *et al.* (2011). All chemicals were of analytical grade and were sourced from Sigma–Aldrich (Tallaght, Dublin, Ireland) unless otherwise stated.

### 2.3.2 Preparation of heated WPI solutions

The WPI was reconstituted (5%, w/v, protein) in ultra-pure water by magnetic stirring at 22°C for 2 h before being adjusted to pH 7.0 with 0.5 M KOH. WPI solutions with either 5.0–30 mM added NaCl, 2.0–30 mM *N*-ethylmaleimide (NEM) or a mixture of 20 mM NaCl and 5 mM NEM were prepared as described above. All solutions were held at 4°C for 18 h before being readjusted to pH 7.0 with 0.5 M KOH at 22°C, as required. Aliquots (5 mL) of each solution were then placed in 10 mL glass tubes (length, 100 mm; external diameter, 12 mm; wall thickness, 2 mm) and heated at 90°C for 30 min in a pre-equilibrated, shaking water bath. Samples were removed and cooled immediately in iced water before being stored at 4°C for further analysis.

### 2.3.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the WPI solutions before and after heating was performed under both reducing and non-reducing conditions in a Tris-HCl buffer (pH 6.8) according to the method of Laemmli (1970) with minor modification as described by Mulcahy *et al.* (2016). Briefly, samples in a reducing sample buffer containing 100 mM dithiothreitol (Pierce, Rockford, IL, USA), were heated at 95°C for 5 min before being loaded (15 µg of protein) into each well of a pre-cast 4-20% gradient acrylamide, 10 x 10 cm, Tris-glycine gel (Pierce) in an AcquaTank mini gel unit (Acquascience, Uckfield, UK). Non-reducing samples were not heated prior to loading as per the manufactures instructions.

### 2.3.4 Whey protein denaturation as determined by pH 4.6-soluble protein

The pH 4.6-soluble fractions of unheated and heated WPI solutions were prepared as described by O'Kennedy and Mounsey (2006) with the following minor modifications. Acetic acid (4 mL of 10%, v/v) was added to each WPI solution (50 mL), which was then heated at 40°C for 10 min, before 4 mL of sodium acetate (1 M) was added, after which each WPI solution was further heated at 40°C for 10 min and allowed to cool to 22°C. The pH of the WPI solutions was readjusted to pH 4.6 with either 10% acetic acid or 1 M sodium acetate (as required), made up to a final volume of 100 mL with ultra-pure water and centrifuged at 10,000 g at 22°C for 20 min. The protein content of the supernatant was determined by the Kjeldahl method (AOAC, 1995) using a nitrogen to protein conversion factor of 6.38. Results are

reported as the pH 4.6 soluble protein (i.e., native protein) expressed as a percentage of the total protein in the solution.

### 2.3.5 Determination of particle size distribution

Particle size distributions of the unheated and heated WPI solutions were determined using dynamic light scattering using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) as described by Mulcahy *et al.* (2016) to evaluate if heating of the protein solutions impacted the particle size distribution. Each solution was diluted 1:100 with ultrapure water, adjusted to pH 6.8 with 0.05 N HCl and allowed to equilibrate at 25°C for 120 s in the cuvette prior to analysis. Detection was completed at a backscattering angle of 173°. The refractive index of protein and water were set at 1.45 and 1.33, respectively, and viscosity was set at 0.89 mPa.s at 25°C. The harmonic hydrodynamic volume-based averaged particle diameter (VMD) in the unheated and heated WPI solutions are reported. The VMD is defined as the average diameter of each peak in the particle size distribution, weighted by the percentage volume distribution under each individual peak, relative to the total area of the distribution as described in ISO:22412.

### 2.3.6 Transmission electron microscopy

Negative staining of the unheated and heated WPI solutions was completed as described by Loveday *et al.* (2010), with the exception that the solutions were diluted to 0.05%, w/v, protein, in ultra-pure water, prior to analysis and not centrifuged or filtered before being stained with 2% uranyl acetate (diluted in ultra-pure water). Specimens were imaged using a JEOL Transmission Electron

Microscope JEM 2000FXII (Jeol Ltd., Tokyo, Japan), operated at 80 kV. Electron micrographs were obtained using a Megaview-III digital camera and AnalySIS software. At least three specimens of each sample were observed to obtain representative micrographs.

### 2.3.7 Determination of available amino groups

#### 2.3.7.1 *O*-phthalaldehyde method

Available amino groups (AAG) were quantified by the *o*-phthalaldehyde (OPA) method as described by Nielsen *et al.* (2001) with minor modifications as detailed by Mulcahy *et al.* (2016). Briefly, the WPI solutions were diluted to 0.1% (w/v) protein with ultrapure water prior to analysis. Absorbance at 340 nm was measured using a Cary 300 Bio UV-visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). Quantification of AAG was performed by reference to an L-leucine standard curve of concentration range 0.2-1.2 mM *versus* absorbance at 340 nm. The concentration of AAG in each WPI solution after 30 min of heating was expressed as a percentage of AAG in the respective unheated solutions.

#### 2.3.7.2 Trinitrobenzenesulfonic acid method

AAG were quantified by the trinitrobenzenesulfonic acid (TNBS) method as described by Spellman *et al.* (2003). Briefly, the WPI solutions were diluted to 0.05% (v/v) protein with sodium dodecyl sulphate (SDS; 1.0%, w/v) prior to analysis. L-leucine (in the concentration range 0.2-1.2 mM) diluted with 1.0%, w/v, SDS was used to construct a standard curve. Absorbance at 340 nm was measured using a Cary 300 Bio UV-visible spectrophotometer (Varian Inc., Palo Alto, CA,

USA). The concentration of AAG in each WPI solution after 30 min of heating was expressed as a percentage of AAG in the respective unheated solutions.

#### 2.3.8 *Statistical analysis*

All analyses were performed in triplicate over three independent trials and mean values  $\pm$  standard deviations are presented. The coefficient of variation (CV) was calculated as the ratio of the standard deviation to the mean and expressed as a percentage. Analysis of variance (ANOVA) was carried out followed by Tukey's mean comparison test to establish the significance of differences among the mean values using the Minitab 16 (Minitab Ltd, Coventry, UK, 2007) statistical analysis package and the level of significance was determined at  $P \leq 0.05$ .

## 2.4 Results and discussion

### 2.4.1 Preliminary experiments on the effects of NaCl and NEM addition on aggregation of whey proteins

Preliminary experiments were performed to investigate the effect of adding different concentrations of NaCl (5-30 mM) or the reducing agent NEM (2-30 mM) to the WPI solutions on the extent of heat-induced whey protein aggregation. It was found that 20 mM NaCl added to the WPI solution prior to heating was effective in inducing protein aggregation upon heating (5%, w/v, protein, pH 7, 90°C for 30 min) without visible clustering of aggregates or the formation of a spatial gel network (results not shown). These results are in keeping with those of Fitzsimons *et al.* (2007) who reported that a WPI solution (3%, w/v, protein, pH 7.0) heated at 80°C for 30 min with  $\leq 25$  mM added NaCl remained non-gelling, whereas, WPI solution containing  $> 25$  mM added NaCl formed a continuous protein gel network on heating.

The reducing agent NEM was added to the WPI solutions at concentrations ranging from 2-30 mM prior to heating at an initial pH of 7.0, at 90°C for 30 min, to determine the minimum concentration required to prevent heat-induced whey protein aggregation. The addition of 5 mM NEM before heating resulted in complete resolution of the monomeric  $\beta$ -lactoglobulin ( $\beta$ -lg) and  $\alpha$ -lactalbumin ( $\alpha$ -lac) bands in non-reducing SDS-PAGE analysis of the heated WPI (Fig. 2.2A, lane 5). This indicated that the addition of 5 mM NEM to the WPI solution was sufficient to inhibit disulfide or sulfhydryl-disulfide mediated interactions between the whey protein molecules during heating. Therefore, WPI solutions containing either 20 mM added NaCl (WPI+NaCl) or 5 mM NEM (WPI+NEM) were selected for the subsequent



analysis together, with a WPI solution containing both 20 mM added NaCl and 5 mM NEM (WPI+NaCl+NEM).

#### 2.4.2 Influence of heating on whey protein denaturation

A high level (84.8%) of native protein was present in the unheated WPI solution with the remaining 15.2% of protein precipitated at pH 4.6 attributed to the presence of a small amount of denatured protein due to the processing conditions (e.g., spray drying) applied during the manufacture of the WPI ingredient. The level of native protein in the WPI solution decreased on heating to 4.4% (Table 2.1) with similar results were reported by Giroux *et al.* (2010) for unheated and heated WPI solutions (1%, w/v, protein, 80°C for 15 min), which contained 85.2% and 2.7% native protein, respectively.

There was no significant ( $P > 0.05$ ) difference between the level of native protein in the heated WPI solution (4.4%) and the heated WPI+NaCl solution (3.9%). However, heating of the WPI+NEM or WPI+NEM+NaCl solution resulted in significantly ( $P \leq 0.05$ ) higher levels of native protein (15.9% and 10.9%, respectively) than that in the heated WPI solution and the heated WPI+NaCl solutions. Croguennec *et al.* (2003) reported similar results for  $\beta$ -lg solution (0.1% protein) containing 0.35 mM NEM, which was heated at 85°C for 24 min; this solution had ~10% less protein precipitated at pH 4.7 compared to the heated  $\beta$ -lg solution without added NEM.

**Table 2.1.** Effect of heating at an initial pH of 7.0 at 90°C for 30 min on the levels of native protein, particle size and concentration of available amino groups, as measured by the o-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods (expressed as a percentage of the concentration of available amino groups in the respective unheated control), of whey protein isolate (WPI) solutions and WPI solutions containing 20 mM added NaCl (WPI+NaCl), 5 mM N-ethylmaleimide (WPI+NEM) or a mixture of 20 mM added NaCl and 5 mM NEM (WPI+NaCl+NEM). Values are presented as mean  $\pm$  standard deviation of data from four independent trials. Means with a common letter are not significantly ( $P > 0.05$ )

Samples	Native Protein	Particle Size	Available Amino Groups	
	pH 4.6 soluble protein	Volume Mean	OPA <sup>(1,2)</sup>	TNBS <sup>(1,2)</sup>
	(% of total protein)	Diameter (nm)		
Unheated WPI	84.8 $\pm$ 0.3	32 $\pm$ 4	100 $\pm$ 1.1 <sup>aA</sup>	100 $\pm$ 0.5 <sup>aA</sup>
Heated WPI	4.4 $\pm$ 0.2	73 $\pm$ 8	94.7 $\pm$ 1.9 <sup>abA</sup>	89.8 $\pm$ 3.1 <sup>bB</sup>
Heated WPI+NaCl	3.9 $\pm$ 0.3	89 $\pm$ 12	91.7 $\pm$ 2.0 <sup>cA</sup>	80.8 $\pm$ 2.9 <sup>cB</sup>
Heated WPI+NEM	15.9 $\pm$ 0.3	63 $\pm$ 17	99.5 $\pm$ 1.8 <sup>abA</sup>	97.5 $\pm$ 2.1 <sup>aA</sup>
Heated WPI+NaCl+NEM	10.9 $\pm$ 0.3	89 $\pm$ 10	95.7 $\pm$ 2.4 <sup>bA</sup>	91.0 $\pm$ 2.1 <sup>bB</sup>

*different from each other.*

<sup>(1)</sup> Means within a column without a common lowercase superscript letter are significantly different ( $P \leq 0.05$ ).

<sup>(2)</sup> Means within a row for the same parameter without a common uppercase superscript letter are significantly different ( $P \leq 0.05$ ).

### 2.4.3 Influence of heating on whey protein aggregation

#### 2.4.3.1 Effect of heating on aggregation of WPI as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis

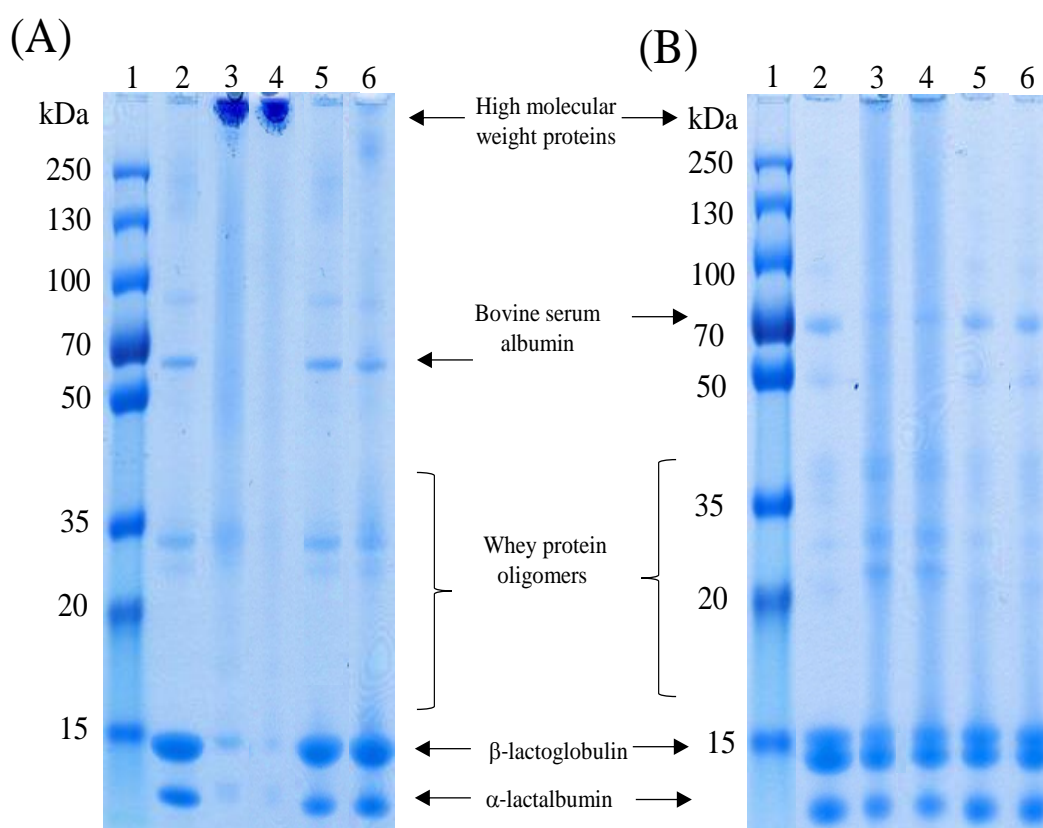
In the unheated WPI solution, the characteristic whey protein bands were evident in the non-reducing and reducing SDS-PAGE gels; these bands represented bovine serum albumin, minor whey proteins including the light and heavy chains of immunoglobulin G, monomers of  $\beta$ -lg and  $\alpha$ -lac, along with whey protein oligomers (Fig. 2.2A and B, lane 2). The heated WPI solution had less monomeric  $\beta$ -lg and  $\alpha$ -lac (Fig. 2.2A, lane 3) resolved in the non-reducing SDS-PAGE gel compared to the unheated WPI solution. A broad range of stain material in the molecular weight range 35-250 kDa was present in the heated WPI sample, along with aggregates (> 250 kDa) which were not resolved by the non-reducing buffer and remained in the loading well of the gel (Fig 6.2A, lane 3). Wijayanti *et al.* (2015) reported a similar electrophoretic pattern for a heated WPI solution (85°C for 30 min) under non-reducing SDS-PAGE conditions; the authors reported the presence of dimers, trimers and oligomers of  $\beta$ -lg and  $\alpha$ -lac in the molecular weight range 35-250 kDa were present, along with material with molecular weight >250 kDa which could not permeate the gel and remained in the well.

The heated WPI+NaCl solution (Fig. 2.2A, lane 4) had a broadly similar electrophoretic pattern to the heated WPI solution without added NaCl (Fig. 2.2A, lane 3) in the non-reducing and reducing SDS-PAGE gel. The heated WPI and WPI+NaCl solution had low levels of monomeric  $\beta$ -lg and  $\alpha$ -lac that resolved compared to the unheated WPI solution and had a range of high molecular weight material, including aggregates (>250 kDa) which did not permeate the non-reducing SDS-PAGE gel and remained in the well (Fig. 2.2A, lane 4). In the non-reducing

gel, the electrophoretic patterns for the heated WPI+NEM and heated WPI+NaCl+NEM solutions were essentially identical to the unheated WPI solution (Fig. 2.2A, lanes 5, 6 and 2, respectively). The presence of NEM (added prior to heating of the WPI solution) prevented the formation of high molecular weight material (>250 kDa) on heating at 90°C for 30 min for the WPI-NEM solution. In the reducing gel, the presence of the reducing agent (50 mM DTT) in the sample buffer improved the resolution of the aggregated proteins; the intensity of the  $\beta$ -lg and  $\alpha$ -lac monomer bands increased considerably for all heated WPI solutions in the reducing gel compared to the intensity of the bands corresponding to these proteins in the same solutions resolved in the non-reducing gel and no large aggregates (>250 kDa) were present (Fig. 2.2B), indicating that the larger aggregates (>250 kDa) were formed *via* disulphide bonding. However, a broad range of stain material (15-250 kDa) remained in the heated WPI and WPI+NaCl solutions in the reducing gel signifying that some aggregates had formed on heating *via* non-covalent interactions.

#### 2.4.3.2 Effect of heating on size distribution of particles in solutions of WPI

Particle size based on dynamic light scattering (DLS) is reported as volume-based mean diameter, as larger particles can dominate the DLS when measured by intensity, therefore volume distributions are considered more appropriate for polydisperse whey protein solutions (Ryan *et al.*, 2012). All unheated and heated WPI solutions displayed bimodal particle size distributions with one large and one very small peak being identified (Fig. 2.3).

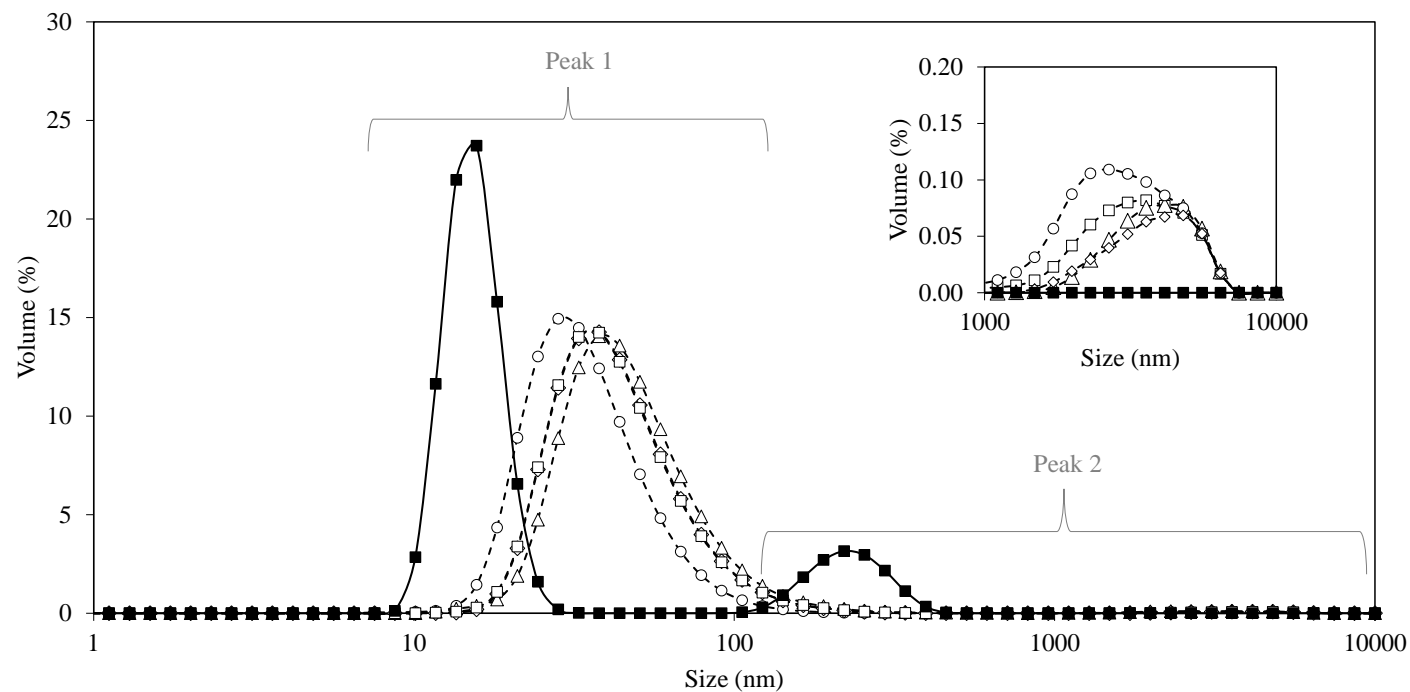


**Fig. 2.2.** One dimensional sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoretograms of whey protein isolate (WPI) solutions and WPI solutions containing 20 mM added NaCl (WPI+NaCl), 5 mM N-ethylmaleimide (WPI+NEM) or a mixture of 20 mM added NaCl and 5 mM NEM (WPI+NaCl+NEM) prepared in a non-reducing (A) and reducing buffer (B). Electrophoretograms were stained for protein with Coomassie Brilliant Blue G-250. The lane identifications for (A) and (B) are as follows: (1) molecular weight marker, (2 and 3) WPI, (4) WPI+NaCl, (5) WPI+NEM and (6) WPI+NaCl+NEM. Lane 2 is an unheated WPI control solution while lanes 3-6 are WPI solutions heated at 90°C for 30 min.

The particles in the unheated WPI solution had a volume-based harmonic mean diameter (VMD) of 32 nm, attributed to the presence of whey protein monomers, dimers, oligomers and small aggregates in the control solution; this increased to 73 nm on heating at 90°C for 30 min, consistent with aggregation of whey proteins (Table 2.1). Similar results were obtained by Loveday *et al.* (2013)

for unheated WPI and gum arabic mixtures where 75% of particles had a VMD between 20 and 30 nm. Although there was no significant difference in the VMD of any of the heated solutions, the heated WPI+NaCl solution had slightly larger particles (89 nm) than that of the WPI solution heated without added NaCl (73 nm) which was attributed to a small amount of very large particles influencing the overall particle size distribution (Fig. 2.3).

The heated WPI solution containing 5 mM NEM had slightly smaller particles (63 nm) than the corresponding peaks in the heated WPI solution and the heated WPI solution containing 20 mM added NaCl (89 nm; Table 2.1). Similar trends were reported by Ryan *et al.* (2012) where the VMD of WPI solutions (3%, w/w, protein) containing 54 mM NaCl increased by ~40 nm when heated at 90°C for 5 min. The heated WPI+NaCl+NEM solution had the same VMD (89 nm) as that of the heated WPI+NaCl solution, which may be attributed to decreased inter-protein charge-based repulsion due to added NaCl which can lead to increased protein-protein interactions, even in the presence of a thiol blocking agent (i.e., NEM) (Matsudomi *et al.*, 1991). Xiong *et al.* (1993) reported that the thermal aggregation of  $\beta$ -lg (1.2 mg/ml) occurred at temperatures  $\geq 76^\circ\text{C}$  due to increased protein-protein interactions; however, the addition of NaCl (0.02-1.0 M) promoted aggregation while the addition of NEM (4-10 mM) decreased protein aggregation in the absence of added NaCl. Interestingly, Xiong *et al.* (1993) reported that a heated  $\beta$ -lg solution with added NEM and NaCl had the same aggregation profile as the  $\beta$ -Lg solution with added NaCl highlighting the importance interactions other than disulphide bonds such as hydrophobic and electrostatic interactions. Brodkorb *et al.* (2015) also suggested that blockage of the disulphide bonds by the addition of NEM may



**Fig. 2.3.** Particle size distributions, by volume, for unheated solutions (closed symbol) and solutions heated at an initial pH of 7.0 at 90°C for 30 min (open symbols) of whey protein isolate (WPI; ■ or □) and WPI solutions containing 20 mM added NaCl (WPI+NaCl; Δ), 5 mM N-ethylmaleimide (WPI+NEM; ○) or a mixture of 20 mM added NaCl and 5 mM NEM (WPI+NaCl+NEM; ◇). Insert shows data for peak 2 with rescaled y- and x-axes.

lead to unfolding of the protein leading to increased hydrophobic interaction that may be further promoted by the addition of NaCl, which brings protein molecules in closer proximity to each other in solution due to decreased electrostatic repulsion.

#### 2.4.4 Effect of heating on microstructure of WPI solutions

In the negatively-stained transmission electron microscopy (TEM) images, the electron beam primarily interacts with the uranyl acetate stain which gathers around the whey protein molecules, i.e., the lighter areas in the TEM images reflect the volume occupied by the whey protein molecules and aggregates). Small, roughly spherical protein particles were present in the unheated WPI solution (Fig. 2.4A) and no obvious differences between micrographs of the unheated WPI solution and of unheated WPI solutions containing NaCl, NEM or the mixture of NaCl and NEM (data not shown). The WPI solution heated at 90°C for 30 min, at pH 7.0 had large rough-edged aggregates composed of discrete globular subunits (Fig. 2.4B), with the subunits being similar to those in the unheated WPI (Fig. 2.4A). Mudgal *et al.* (2010) reported similar results for negatively stained TEM micrographs of heated  $\beta$ -lg solutions (pH 7.0, heated at 85°C for 3 h) where repeating globular structural subunits were visibly interconnecting to form a larger network of aggregates.

Heating of the WPI+NaCl solution resulted in larger, rough-edged protein aggregates which appeared to have denser centres (i.e., less globular subunits visible; Fig. 2.4C) than the aggregates formed in the WPI solutions heated without added NaCl. Walkenström *et al.* (1998) performed TEM microscopy of gelled WPI systems (heated at 85°C for 3 h) with 50 mM added NaCl and reported that roughly spherical globular subunits were visible which formed elongated particulate strands. The

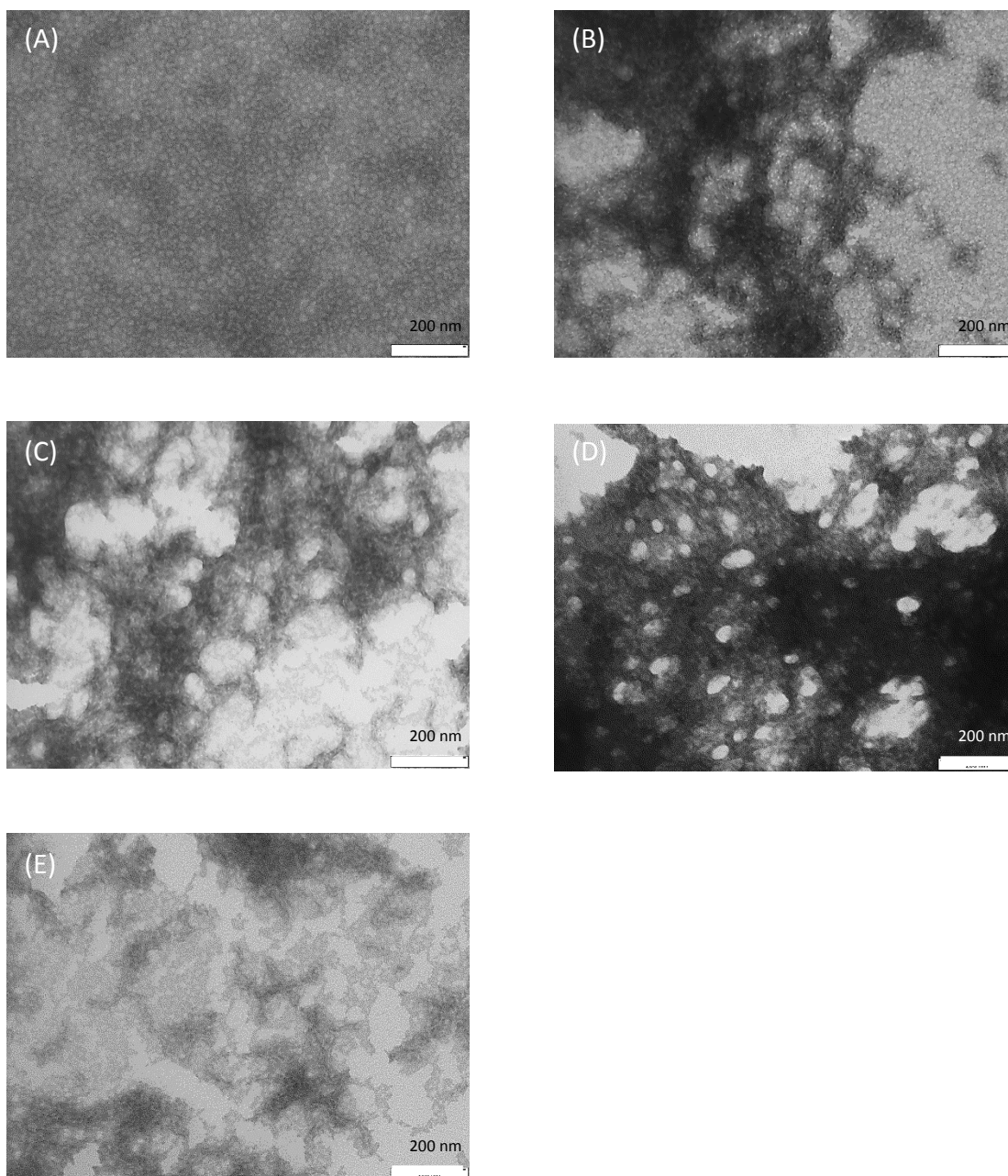


heated WPI+NEM solution had smaller aggregates (Fig. 2.4D) than the heated WPI solution and some subunits were still visible, indicating that the presence of 5 mM NEM in the WPI solution on heating partially inhibited the formation of larger aggregates compared to the heated WPI solution (Fig. 2.4B).

Heating of the WPI+NaCl+NEM solution resulted in heterogeneous structures with some aggregates having a closely compacted arrangement, with no subunits visible, while other aggregates retained their discrete globular subunit structures (Fig. 2.4E). Bryant and McClements (1998) reported that protein-protein interactions in whey protein solutions were promoted in a high ionic strength environment as bonds can form at several points on the surface of the protein molecules (due to a reduction in the electrostatic repulsion) leading to the formation of large, roughly spherical, whey protein aggregates. Furthermore, Fitzsimons *et al.* (2007) and Verheul *et al.* (1998) reported that in heated WPI/ $\beta$ -lg solutions, the level of protein denaturation can be similar while the rate and extent of aggregation may vary depending on the presence of added NaCl.

#### 6.4.4 *Influence of whey protein aggregation on the level of available amino groups as measured by OPA and TNBS methods*

The heated WPI solution had 3.3% and 10.2% lower levels of AAG, as measured by the OPA and TNBS methods, respectively, compared to the unheated WPI control (Table 2.1). Chevalier *et al.* (2001), using the OPA method, reported similar results for  $\beta$ -lg solutions (0.4% protein) heated at 60°C for 72 h, which had ~5.0% lower AAG compared to the unheated  $\beta$ -lg solutions.



**Fig. 2.4.** Negative stained transmission electron micrographs of an unheated whey protein isolate (WPI) solution (A), WPI solution heated at an initial pH of 7.0 at 90°C for 30 min (B) and heated WPI solutions containing 20 mM added NaCl (WPI+NaCl; C), 5 mM N-ethylmaleimide (WPI+NEM; D) or a mixture of 20 mM added NaCl and 5 mM NEM (WPI+NaCl+NEM; E). The scale bar = 200 nm.

The significantly ( $P \leq 0.05$ ) lower level of AAG in the heated WPI solution, as measured by the TNBS method compared with the OPA method, may be predominantly attributed to aggregation of the whey protein molecules *via* covalent and non-covalent interactions (Table 2.1, Figs. 2.1 and 2.4B). This was likely to have resulted in the amino groups being located within a compact aggregated protein matrix, rendering them unavailable for reaction with the TNBS molecules, or the reaction of the whey proteins with low levels of residual lactose present in the WPI ingredient. To a lesser extent, localised charge modification of the amino groups in the whey proteins due to thermal treatment may lead to a lower response of the TNBS reagent to AAG in the heated WPI solutions compared to the unheated control as the TNBS molecule reacts with amino groups *via* a bimolecular aromatic nucleophilic substitution (Fig. 2.1B) that requires the nucleophile (e.g., lysine) to be unprotonated (i.e.,  $R-NH_2$ ) (Cayot & Tainturier, 1997).

Means and Feeney (1995) reported that exposure of charged groups (such as those exposed on heating) adjacent to AAG within the protein structure can influence the rate of reaction of the TNBS reagent; with an increased rate observed with positively charged adjacent groups and a decreased rate with negatively charged adjacent groups (Fig. 2.5). The observed reduction in the response of the TNBS reagent in the WPI solution on heating may also be attributed to exposure of previously buried sulfhydryl groups within proteins as the TNBS reagent can react with free sulfhydryl groups instead of the amino groups, albeit at a slower rate than with amino groups, to form a non-UV-visible product (Kotaki *et al.*, 1964; Spellman *et al.*, 2003). Jacobs *et al.* (1986) reported that reactivity of the OPA reagent to amino groups was found to be influenced by a number of experimental parameters

including thiol structure, thiol concentration, amino structure, solvent composition, and pH; however, in the current study the level of AAG in the heated WPI solution was not significantly ( $P > 0.05$ ) different to that in the unheated WPI solution when measured by the OPA method, indicating that the OPA method is less impacted than the TNBS method by conformational changes of whey protein molecules on heating.

The heated WPI+NaCl solution was the only solution that had significantly ( $P \leq 0.05$ ) lower levels of AAG (91.7% and 80.8%, respectively) when measured by both the OPA and TNBS methods compared to the unheated WPI solution (Table 2.1). There was an 8.3% reduction in AAG in the heated WPI+NaCl solution, compared to the unheated WPI solution, when measured by the OPA method and a 19.2% reduction in AAG when measured using the TNBS method. The added NaCl in the heated WPI+NaCl solution resulted in a reduction in the electrostatic repulsions between the whey protein molecules, which consequently allowed greater interaction of the protein molecules on heat treatment, leading to the formation of larger, more dense protein aggregates (Table 2.1, Figs 6.4D, Fig. 2.5). The reduction in the levels of AAG in the heated WPI+NaCl solution, as measured by the OPA and TNBS methods, is most likely due to fewer amino groups being available to react with the OPA and TNBS reagents as access to amino groups within the compact, aggregated protein matrix would have been hindered. Bulky aggregated protein structures have been reported to decrease the rate of reaction of the OPA reagent with the thiol group in the OPA reagent buffer which can reduce the formation of the UV-absorbing isoindole product (Chen *et al.*, 1979).

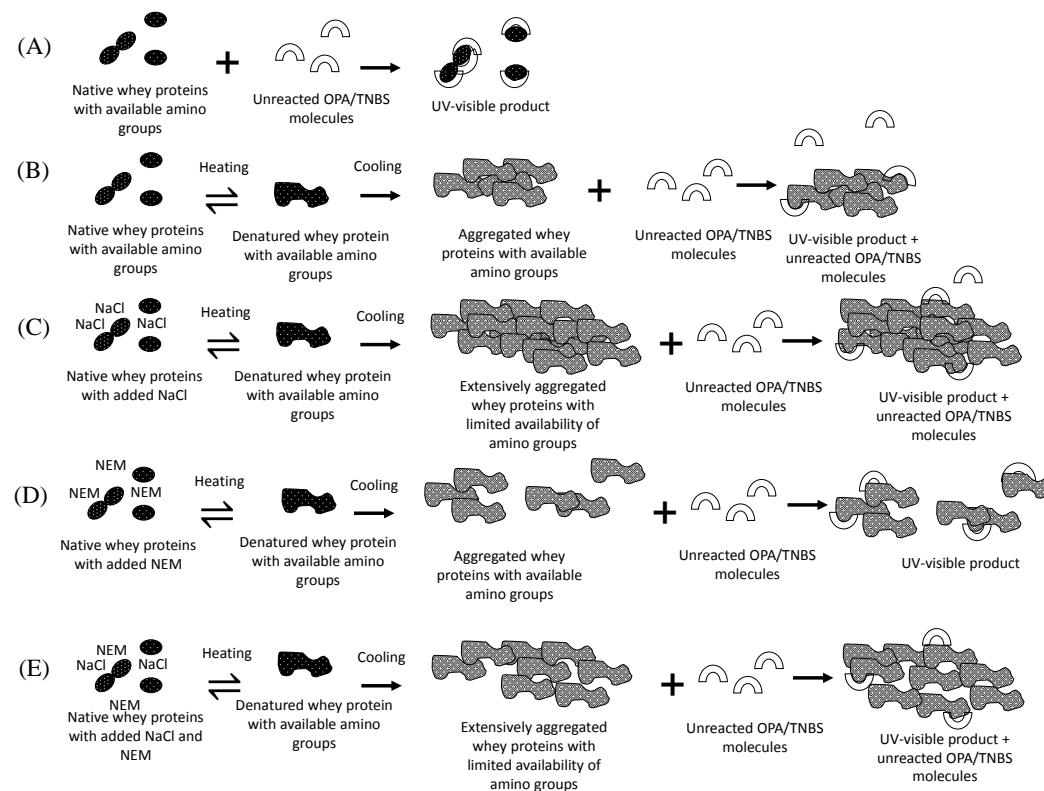
There was no significant ( $P > 0.05$ ) difference in the level of AAG in the heated WPI+NEM solutions and the unheated WPI control solution when measured

by either the OPA or TNBS methods (Table 2.1). The smaller protein aggregates (Table 2.1, Figs 6.2A and 6.4D) present in the heated WPI+NEM solution, compared to all other heated WPI solutions, was attributed to the disruption of the intramolecular disulphide bonds due to the presence of NEM, which in turn, may have resulted in the amino groups remaining more available for reaction with the OPA and TNBS molecules. Similar results were reported by Wijayanti *et al.* (2014b) for heated WPI solutions (pH 7.0, 85-97.5°C, for 30-150 min) containing NEM which were reported to have lower levels of protein aggregation, as determined by size exclusion HPLC and SDS-PAGE, compared to heated WPI control solution (i.e., without NEM), as the NEM blocked the reactive thiol groups in the whey protein molecules, thus limiting protein aggregation.

There was a 4.3 and 9.0% reduction in the levels of AAG in the heated WPI+NaCl+NEM solution as measured by the OPA and TNBS methods, respectively, compared to the unheated control. The heated WPI+NaCl+NEM solution had a size distribution profile similar to that of the heated WPI+NaCl solution (i.e., without NEM), which indicated that a decrease in inter-protein charge repulsion, due to the presence of added NaCl, can lead to increased protein-protein interactions, even in the presence of a thiol-blocking agent. Although the presence of NEM would have limited disulfide bonding in the heated WPI solutions, aggregation still occurred *via* non-covalent bonding which is promoted by the presence of NaCl on heating of whey protein solutions (Sawyer, 1968; Wijayanti *et al.*, 2015).

A comparison of the coefficient of variation ( $n = 12$ ) of the OPA method (2.3%) and the TNBS method (4.7%) indicated that the OPA method was more

reproducible than the TNBS method. Morales *et al.* (1995) reported a coefficient of variation of 2.0% ( $n = 10$ ) for available lysine in pasteurised milk when using the OPA method and Obi (1982) reported a coefficient of variation of 6.5% ( $n = 90$ ) for available lysine in maize seed when using the TNBS method. Adler-Nissen (1979) reported that several factors can contribute to the greater coefficient of variation for the TNBS method, including the presence of insoluble protein material which can result in errors during spectrophotometric analysis and the TNBS reagent reacting slowly with hydroxyl ions in solution which can cause baseline drift. Overall, the levels of AAG in all heated WPI, WPI+NaCl, WPI+NEM and WPI+NaCl+NEM solutions were consistently, though not always significantly ( $P > 0.05$ ), lower when measured by the TNBS method, compared with the OPA method. This would indicate that when measuring the level of AAG, the TNBS method was impacted more by heat-induced conformational changes of the whey protein molecules (i.e., aggregation) than the OPA method.



**Fig. 2.5.** Diagrammatic representation of heat-induced aggregation of whey proteins and the influence of aggregation on the availability of amino groups as measured by the *o*-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods. The diagram represents unheated, native whey proteins (A); aggregated whey proteins on heated at an initial pH of 7.0 at 90°C for 30 min (B), aggregated whey proteins containing 20 mM added NaCl (C), aggregated whey proteins containing 5 mM *N*-ethylmaleimide (D) and aggregated whey proteins containing a mixture of 20 mM added NaCl and 5 mM NEM (E). The differentiation between hydrophobically associated aggregates and disulphide-linked aggregates due to the presence of NaCl and NEM is represented by the size of the aggregates formed on heating.

## 2.5 Conclusion

The results of this study demonstrate that the OPA method for measuring AAG is less impacted than the TNBS method to heat-induced structural changes of whey protein molecules, in particular, sulfhydryl-disulfide interchange and disulphide-bond mediated aggregation. The levels of AAG in all heated WPI solutions as measured by the TNBS method were consistently, though not always significantly ( $P > 0.05$ ), lower than those measured by the OPA method. This lower level of AAG in the heated WPI solutions is attributed to the amino groups being located within large, dense protein aggregates, hence being rendered unavailable for reaction with the OPA and TNBS molecules. Overall, these results provide a systematic evaluation of the effects of heat-induced aggregation of whey proteins on the availability of amino groups as measured by the OPA and TNBS methods. Both the OPA and TNBS methods can also be used to quantify reactions involving the modification of AAG including succinylation (El-Adawy, 2000; Wierenga *et al.*, 2005), methylation (Kosters *et al.*, 2003), conjugation (Mulcahy *et al.*, 2016, Chapter 2), hydrolysis (Spellman *et al.*, 2003) and thiolation of proteins (Broersen *et al.*, 2006) and the findings herein may have implications for those measuring the consumption or liberation of AAG in such studies.

## 2.6 Acknowledgements

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## Chapter 3

### *Physicochemical properties of whey protein conjugated with starch hydrolysis products of different dextrose equivalent values*

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### 3.1 Abstract

The influence of dextrose equivalent (DE) values of starch hydrolysis products on the physicochemical properties of whey protein during conjugation by the Maillard reaction was determined. Starch hydrolysis products (maltodextrin; MD, corn syrup solids; CSS, or glucose; 5% carbohydrate) were incubated with whey protein isolate (WPI; 5% protein) at an initial pH of 8.2 and 90°C for up to 24 h. The extent of conjugation increased considerably with increasing DE value of the starch hydrolysis products. At 8 h of heating, WPI solutions containing MD or CSS had levels of advanced Maillard reaction products (2.7-3.8 relative fluorescence units; RFU), which were substantially lower than those in WPI-glucose solutions (6.3 RFU). All samples displayed increases in particle size on heating at 90°C, with the DE value of the starch hydrolysis product influencing the size of particles formed; in the glucose-containing sample, there was no formation of large ( $>271 \pm 28$  nm average volume diameter) particles. It was concluded that optimal conjugation levels were achieved for solutions containing MD or CSS ( $DE \leq 38$ ) after heating for 8 h, conditions which resulted in the greatest reduction in available amino groups and minimal production of advanced Maillard reaction products and colour.

## 3.2 Introduction

Heat treatment is an essential unit operation in the production of many commercial food products to ensure microbiological safety and quality. Food proteins, in the presence of a reducing carbohydrate, can undergo a series of complex chemical changes during heating, known collectively as the Maillard reaction. Hodge (1953) simplified the Maillard reaction into three separate stages. The early stage consists of the condensation reaction between  $\epsilon$ - or  $\alpha$ -amino and carbonyl groups, formation of the Schiff base and irreversible Amadori rearrangement leading to the formation of Amadori products. It is at this stage that conjugation occurs, where a covalent linkage is formed between protein and carbohydrate (Liu *et al.*, 2012). The intermediate stage involves a range of reactions, including cyclisations, dehydrations, retro-aldolisations, re-arrangements, isomerisations and further condensations, which result in degradation of amino acids and carbohydrates (Ames, 1998). The later stage involves the production of a range of advanced Maillard reaction products (AMP), nitrogenous polymers and co-polymers, known collectively as melanoidins (Hodge, 1953). These advanced stages are complex and variable, depend on the reaction conditions, and involve dehydration and decomposition of the early reaction products (Ames, 1998). Several intrinsic and extrinsic factors, such as nature, concentration and ratio of the reactants (amino and carbonyl compounds), temperature, time, pH and water activity influence the yield and type of Maillard reaction products (Liu *et al.*, 2012).

Starch hydrolysis products such as maltodextrin (MD), corn syrup solids (CSS) and glucose are used extensively in the food industry as stabilisers, viscosity modifiers, energy sources, bulking agents and sweeteners. MD and CSS consist

of D-glucose units linked by  $\alpha$ -(1-4) and  $\alpha$ -(1-6) glycosidic bonds in chains of variable length and are classified according to their dextrose equivalent (DE) value, a measure of the total reducing power of the carbohydrates present (Chronakis, 1998). MDs are starch hydrolysis products having DE values  $<20$ , while CSS have DE values  $\geq 20$  (Pancoast & Junk, 1980). Starch hydrolysis products such as MD and CSS are typically composed of a mixture of molecules with different chain lengths, with variations in chain length related to their DE value, i.e., the shorter the average chain length, the higher the DE value (Dziedzic & Kearsley, 1995).

Whey protein ingredients are commonly used in foods to harness their nutritional and functional properties. However, whey proteins are susceptible to heat-induced denaturation, phase separation, increased turbidity and impairment of emulsification properties when subjected to thermal processes (LaClair & Etzel, 2010; Simmons *et al.*, 2007). Aggregation and interactions between proteins and other ingredients (e.g., minerals and emulsion interfacial material) can limit the amount of whey protein used in the formulation of foods due to increased viscosity and gelation during thermal processing or subsequent storage. Controlling the size of heated whey protein particles prior to incorporation into formulations, has been shown to improve the heat stability and reduce the viscosity of protein dispersions compared to using native whey proteins (Sağlam *et al.*, 2014). Conjugation of proteins *via* the Maillard reaction is a growing area of interest, with several studies completed on the use of conjugation to modify physicochemical and functional properties, including solubility, thermal stability, water-binding capacity, emulsifying properties, antioxidant activity and allergenicity of food proteins

(Akhtar & Dickinson, 2007; Chen *et al.*, 2013; Niu *et al.*, 2011; O'Regan & Mulvihill, 2009; Rondeau *et al.*, 2010; Zhang *et al.*, 2014; Zhu *et al.*, 2010).

Previously, some studies have focused on conjugation using pure whey protein fractions, such as  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin (Jiang & Brodkorb, 2012; Zhang *et al.*, 2014) and bovine serum albumin (Huang *et al.*, 2013) and many of the studies have used dry heating approaches to induce conjugation (Li *et al.*, 2005; Wang & Ismail, 2012; Zhang *et al.*, 2014). Böttger *et al.* (2013) and Zhu *et al.* (2010) studied the physicochemical and functional properties of whey protein isolate conjugated with dextran in a heated aqueous environment (60 °C for 24 h); however, published scientific reports on the physicochemical properties of whey proteins conjugated with a range of starch hydrolysis products in a heated aqueous environment appear to be limited.

The present study focused on a systematic evaluation of the influence of starch hydrolysis product chain-length on the rate and extent of conjugation to whey proteins during heating. The conditions used in this study were chosen as it is economically desirable to use shorter processing times at higher temperatures and it is easier to control the extent of the Maillard reaction in a heated aqueous environment compared with when dry heat is applied to induce conjugation, as previously described by Zhu *et al.* (2008). While it is desirable to achieve conjugation in the early stages of the Maillard reaction, it is also beneficial to limit the progression of the Maillard reaction to advanced stages, as AMP are largely responsible for some of the less desirable aspects of the Maillard reaction (e.g., off-flavours, loss of nutritional value, protein crosslinking and generation of potentially toxic compounds). The objective of using a reaction time course of up to 24 h was

to generate a better understanding of the progression of the Maillard reaction while minimising the production of AMPs.

### 3.3 Materials and Methods

#### 3.3.1 Materials

Whey protein isolate (WPI), BiPro<sup>®</sup>, containing 89.4% protein (Kjeldahl method, AOAC, 1995), 3.0% ash (heated at 500°C until white ash obtained; AOAC, 1995), 4.5% moisture (oven drying; IDF, 1987a) and 0.4% fat (Rose Gottlieb method; IDF, 1987b) was obtained from Davisco Foods International (Le Sueur, MN, USA). Lactose level (0.3%) was determined by ion-exchange high-performance liquid chromatography as described by Crowley *et al.* (2015). Maltodextrin (MD) and corn syrup solids (CSS) with dextrose equivalent (DE) values of 6, 12, 17, 30 or 38 were obtained from Corcoran Chemicals Ltd. (Dublin, Ireland). Glucose (DE 100) and all other chemicals were of analytical grade and were sourced from Sigma–Aldrich (Dublin, Ireland). All MD, CSS and glucose ingredients had moisture content of 4.7–5.0% and ash content of 0.1–0.2%.

#### 3.3.2 Preparation of WPI-carbohydrate conjugates

Blends of WPI (5%, w/v, protein) and carbohydrate (5%, w/v), made up of MD or CSS with DE values of 6, 12, 17, 30 or 38, or glucose (DE 100), were prepared in ultrapure water containing 0.02% sodium azide and allowed to solubilise for 2 h at 22°C with low speed magnetic stirring. The solutions were adjusted to pH 8.2 with 0.5 N KOH and allowed to hydrate for 18 h at 4°C, before being readjusted to pH 8.2 with 0.5 N KOH at 22°C. Aliquots (250 mL) of each solution were then placed in 500 mL screw-capped, glass, conical flasks and heated at 90°C for up to 24 h in a

pre-equilibrated, shaking water bath. Samples were removed at 3, 5, 8 and 24 h and cooled immediately in iced water and stored at 4°C for further analysis. A control solution of WPI (5%, w/v, protein) was prepared, heated and sampled as described above.

### 3.3.3 Determination of available amino groups

Available amino groups were quantified by the *o*-phthalaldehyde (OPA) method as described by Nielsen *et al.* (2001). The WPI-carbohydrate solutions were diluted to 0.1% (w/v) protein with ultrapure water prior to analysis. Absorbance at 340 nm was measured using a Cary 300 Bio UV-visible spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA). The values of available amino groups were obtained by reference to an L-leucine standard curve of concentration 0.2-1.2 mM versus absorbance at 340 nm. The concentration of available amino groups in each WPI and WPI-carbohydrate solution at 3, 5, 8 and 24 h of heating was expressed as a percentage of available amino groups in the respective unheated solutions.

### 3.3.4 Determination of Schiff base and furosine

WPI-carbohydrate solutions were diluted to 0.05% (w/v) protein with ultrapure water and absorbance scans of samples after 0, 3, 5, 8 and 24 h of heating were carried out in the UV-visible range of 200 to 800 nm, with a Cary 300 Bio UV-visible spectrophotometer. The extent of conjugation was assessed by the absorbance of Schiff base at 304 nm as described by Zhu *et al.* (2008).

Furosine (FUR) in WPI and WPI-carbohydrate solutions heated at an initial pH of 8.2 at 90°C for 8 h and their respective unheated controls was quantified by ion-pair reverse-phase high-performance liquid chromatography (RP-HPLC) as described by the ISO (2004) method. Briefly, FUR was extracted from the samples by acid hydrolysis; 6 mL of 10.6 N HCl was added to the sample (2 mL) in a vial before the vial was hermetically sealed and heated at 110°C for 23 h. Directly before RP-HPLC analysis, the hydrolysed sample was filtered through a 500 mg solid phase extraction (SPE) column (Discovery DSC-18, Supelco, Sigma–Aldrich, Dublin, Ireland) previously conditioned with 5 mL of methanol and 10 mL of ultra-pure water and the FUR was eluted with 3 mL of 3 M HCl. Separation was achieved by injecting the sample (20  $\mu$ L) onto a dedicated FUR column (Luna C8, 5  $\mu$ m, 4.6 x 250 mm, 100Å; Phenomenex, Torrence, CA) at a flow rate of 0.5 mL min<sup>-1</sup> which was maintained at 35°C. Each measurement lasted 7 min and the column eluate was monitored at 280 nm. The isocratic mobile phase consisted of 0.4% acetic acid in ultra-pure water. An 8 point standard curve of FUR was constructed ranging from 50  $\mu$ g L<sup>-1</sup> to 400  $\mu$ g L<sup>-1</sup> for quantification.

### 3.3.5 Fluorescence of advanced Maillard reaction products and soluble tryptophan

Fluorescence of advanced Maillard reaction products (F<sub>AMRP</sub>) and soluble tryptophan (F<sub>Trp</sub>) were measured as described by Birlouez-Aragon *et al.* (2002). Fluorescence of each solution was measured using a Kontron SFM-25 spectrofluorometer (Jobin Yvon, France) equipped with a xenon lamp at excitation/emission wavelengths of 290/340 nm for tryptophan (F<sub>Trp</sub>) and 330/420



nm for advanced Maillard reaction products (FAMRP) and expressed in relative fluorescence units (RFU).

### 3.3.6 *Measurement of colour*

The colour of each solution was measured using a Minolta Chroma Meter CR-colorimeter 400 (Minolta Ltd., Milton Keynes, UK) using CIE colour chromaticity coordinates and expressed as  $L^*$  (light-dark) values. Each solution was loaded into a glass cell (CM-A98, optical path length: 10 mm) which was held in position by means of a transmittance specimen holder (CM-A96) and positioned with a calibration plate behind the glass cell, before  $L^*$  value was measured.

### 3.3.7 *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed according to Laemmli (1970) with the following modifications: WPI and WPI-carbohydrate solutions (10  $\mu$ L), before and after 8 h of heating, were incubated with 40  $\mu$ L of a reducing sample buffer containing 0.3 M Tris-HCl (pH 6.8), 5% SDS, 50% glycerol and 100 mM dithiothreitol (Pierce, Rockford, IL, USA), before being heated at 95°C for 5 min prior to loading a volume equivalent to 20  $\mu$ g of protein into each gel well of a pre-cast 4-20% acrylamide, 10 x 10 cm, tris-glycine gel (Pierce, Rockford, IL, USA) in an AcquaTank mini gel unit (Acquascience, Bellbrook Industrial Estate, Uckfield, UK). Following separation by electrophoresis at a constant 150 volts for ~2.5 h, the gel was removed from the electrophoresis unit, fixed in 5:1:4 methanol:acetic acid:water, and stained for protein (18 h at 22°C) with Coomassie brilliant blue R-

250 (O'Regan & Mulvihill, 2009). Gels were destained in 4:1:5 methanol:acetic acid:water until a clear background was obtained. Carbohydrate staining was performed on the gels using the periodic acid-Schiff method (Glycoprotein Staining Kit, Pierce Rockford, IL, USA) as per manufacturer's instructions to visualise the migration of conjugated proteins.

### 3.3.8 Particle size distribution

The particle size distribution of the WPI and WPI-carbohydrate solutions heated at an initial pH of 8.2 at 90°C for 8 h and their respective unheated controls was measured to evaluate if heating, and the covalent attachment of different chain-length starch hydrolysis products during conjugation, impacted the particle size distribution of the solutions. The size of particles in the selected solutions was measured by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). Each solution was diluted 1:100 with ultrapure water, adjusted to pH 6.8 with 0.05 N HCl and allowed to equilibrate at 25°C for 120 s in the cuvette prior to analysis. Detection was completed at a backscattering angle of 173°. The refractive index of protein and water were set at 1.45 and 1.33, respectively, and viscosity was set at 0.89 mPa.s at 25°C. The overall volume based mean diameter (VMD) of all solutions is reported along with the percentage area and VMD of individual peaks in the particle size distribution profile.

### 3.3.9 Statistical analysis

The preparation of all solutions and their analysis was completed in three independent trials. Analysis of variance (one-way ANOVA) was carried out

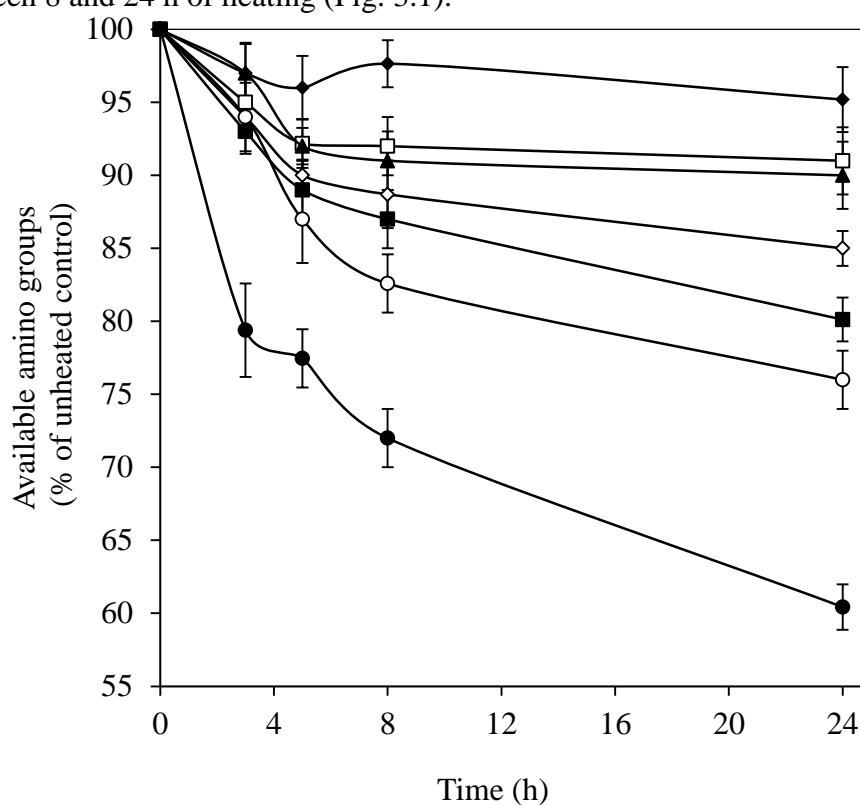
followed by Duncan's mean comparison test to establish the significance in differences among the mean values using the Minitab 16 (Minitab Ltd, Coventry, UK, 2007) statistical analysis package and the level of significance was determined at  $P \leq 0.05$ .

### 3.4 Results and Discussion

#### 3.4.1 Available amino groups

During the early stages of the Maillard reaction,  $\alpha$ -amino groups of proteins/peptides and  $\epsilon$ -amino groups of lysyl residues react with the carbonyl functional groups of reducing sugars. This results in a reduction of available amino groups and can be used to compare the reactivity of different carbohydrate ingredients during conjugation (Laroque et al., 2008). In the WPI control, the level of available amino groups was reduced by 4.8% on heating at 90°C for 24 h (Fig. 3.1). This limited decrease in available amino groups is most likely due to heat-induced reaction of whey protein with low levels of lactose (0.3%) present in WPI or structural changes within the protein causing blockage of available amino groups (Nacka *et al.*, 1998). Similar results were obtained by Chevalier *et al.* (2001), who reported that  $\beta$ -lactoglobulin ( $\beta$ -lg), heated at 60°C for 72 h, reduced in available amino groups by 5% compared to native  $\beta$ -lg possibly due to structural modification of the protein. The dextrose equivalent (DE) value of maltodextrin (MD), corn syrup solids (CSS) and glucose (DE 100) affected the rate and extent of the reduction of available amino groups in WPI during heating of WPI-MD/CSS/glucose solutions. The reactivity of the different starch hydrolysis products decreased in the following order; glucose DE 100  $\gg$  CSS DE 38 > CSS DE 30 > MD DE 17 > MD DE 12 >

MD DE 6. The rate of the Maillard reaction is related to the amount of the reducing carbohydrate present in the open, short chain form; the higher this quantity, the faster the Maillard reaction will occur (Chevalier *et al.*, 2001; van Boekel, 2001). In addition to having fewer carbonyl groups, it has been suggested that steric hindrance of higher molecular weight ( $M_w$ ) starch hydrolysis products would cause lower conjugation rates on reaction with protein (ter Haar *et al.*, 2011). In this study, the greatest reduction in available amino groups occurred during the first 8 h of heating, with a considerably lesser decrease in available amino groups for all samples between 8 and 24 h of heating (Fig. 3.1).

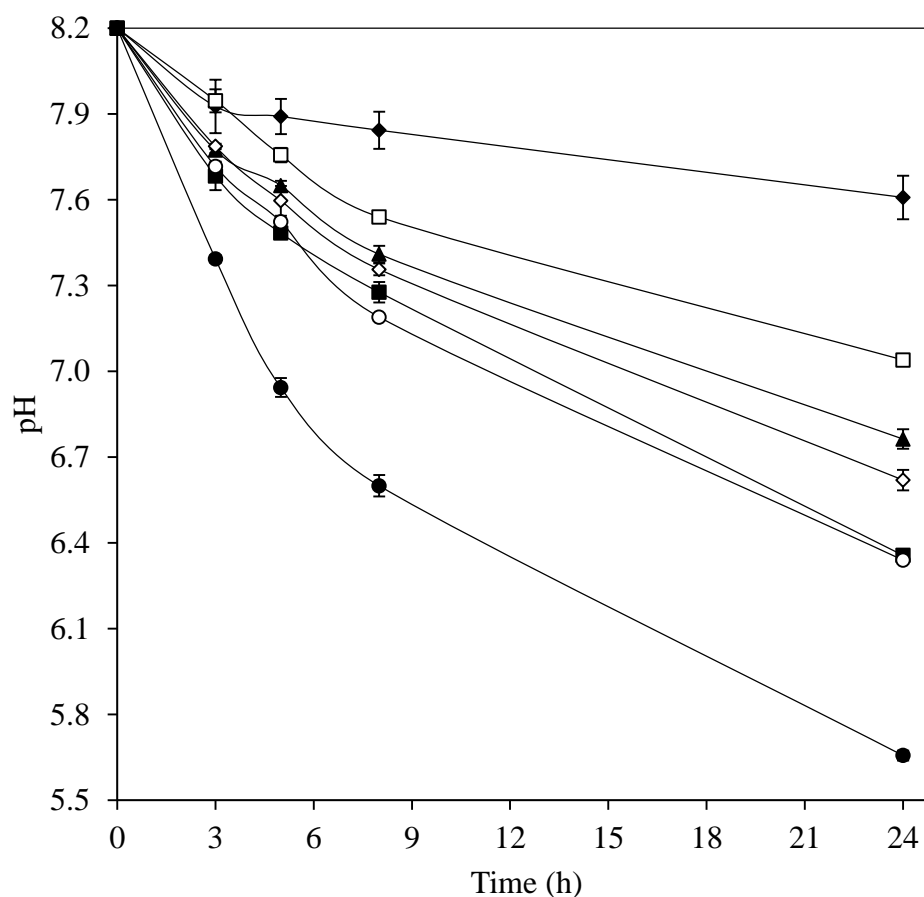


**Fig. 3.1.** Concentration of available amino groups in whey protein isolate (WPI) and WPI-carbohydrate solutions containing maltodextrin (MD), corn syrup solids (CSS) or glucose of different dextrose equivalent values from 6 to 100 heated at an initial pH of 8.2 at 90°C for 24 h; WPI (◆), WPI-MD6 (□), WPI-MD12 (▲), WPI-MD17 (◇), WPI-CSS30 (■), WPI-CSS38 (○) and WPI-glucose (●).

### 3.4.2 Change in pH

The pH of WPI and WPI-carbohydrate solutions decreased significantly ( $P \leq 0.05$ ) during heating (Fig. 3.2). Preliminary trials demonstrated that using an initial pH of 8.2 was effective at promoting conjugation in WPI-MD/CSS solutions while ensuring that solutions were close to neutral pH (7.2-7.5) after 8 h of heating at 90°C, minimising the requirement for subsequent acid/base addition for neutralisation. Furthermore, the initial pH of 8.2 was chosen to maintain pH >7 at 8 h of heating at 90°C for the WPI-MD/CSS solutions, thereby minimising the production of furfural and hydroxymethylfurfural (HMF) which have been linked to potential toxicity and carcinogenicity in rat models (Husøy *et al.*, 2008).

Intermediate-stage Amadori products are degraded by different reaction pathways depending on pH; for example, at pH  $\leq 7$ , degradation takes place *via* the 1, 2-enolization pathway which favours the formation of furfural or HMF. At pH >7, the degradation of Amadori products involves the 2,3-enolization pathway, favouring the production of reductones and fragmentation products such as hydroxyacetone and 2, 3-butanedione, therefore minimising HMF formation (Ames, 1998; Liu *et al.*, 2012). With decreasing carbohydrate chain-length, there was an increase in the rate and extent of pH change in WPI-MD/CSS/glucose solutions during heating. The pH of the WPI-CSS38 solution decreased from 8.2 to 6.3 after heating at 90°C for 24 h; this result was similar to those of Jiang and Brodkorb (2012), who reported that  $\beta$ -lactoglobulin and ribose heated at 95°C for 5 h showed a reduction in pH from 8.4 to 5.3. In accordance with the reduction in available amino groups, the pH decreased most rapidly during the first 8 h of heating for all solutions.

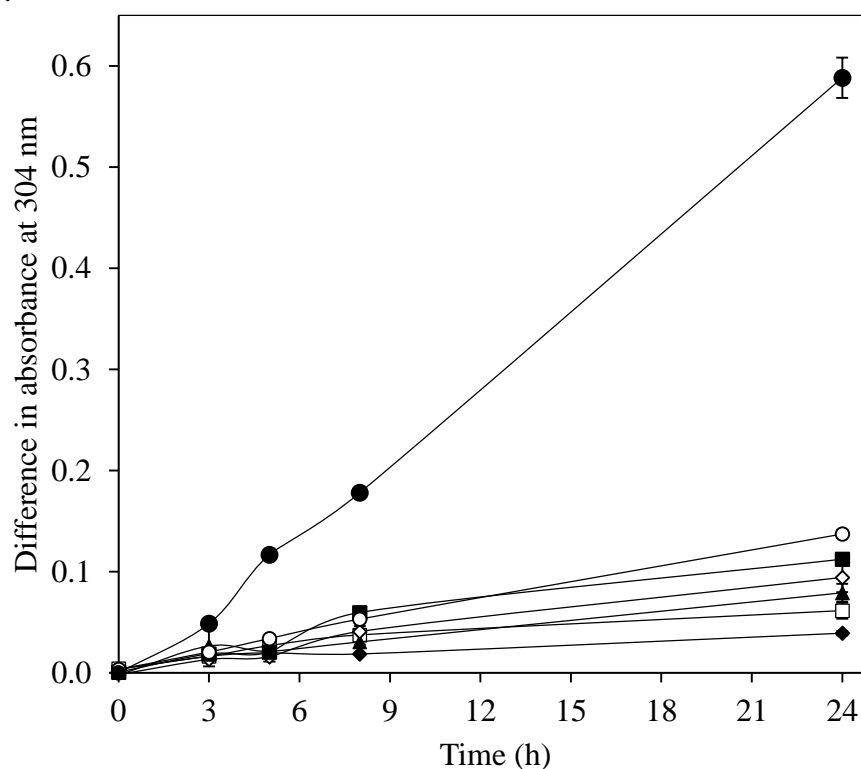


**Fig. 3.2.** pH of whey protein isolate (WPI) and WPI-carbohydrate solutions containing maltodextrin (MD), corn syrup solids (CSS) or glucose of different dextrose equivalent values from 6 to 100 heated at an initial pH of 8.2 at 90°C for 24 h; WPI (◆), WPI-MD6 (□), WPI-MD12 (▲), WPI-MD17 (◇), WPI-CSS30 (■), WPI-CSS38 (○) and WPI-glucose (●).

The pH of solutions continued to decrease with increasing heating time after 8 h while the reduction in available amino groups was minimal; this is indicative of a greater production of advanced Maillard reaction products (AMRP) relative to conjugation on heating for >8 h. The reduction of pH during the Maillard reaction is attributed to the formation of organic acids, such as formic and acetic acid, and the consumption of charged amino acids, such as the basic amino acid lysine (Ames, 1998; Brands & van Boekel, 2002; Lertittikul *et al.*, 2007).

### 3.4.3 Development of Schiff base and furosine on heating

Zhu *et al.* (2008) showed that the early stages of the Maillard reaction can be monitored by measuring the development of Schiff base which is formed by the condensation reaction of reducing sugars with free amino groups. The production of the Schiff base conjugation product in WPI and WPI-carbohydrate solutions as a function of heating time, at 90°C for up to 24 h is shown in Fig.3.3. The levels of Schiff base in all solutions increased during heating and all WPI-carbohydrate solutions had higher levels of Schiff base than the WPI control at 8 h of heating at 90°C.



**Fig. 3.3.** Development of Schiff base in whey protein isolate (WPI) and WPI-carbohydrate solutions containing maltodextrin (MD), corn syrup solids (CSS) or glucose of different dextrose equivalent values from 6 to 100 heated at an initial pH of 8.2 at 90°C for 24 h; WPI (◆), WPI-MD6 (□), WPI-MD12 (▲), WPI-MD17 (◇), WPI-CSS30 (■), WPI-CSS38 (○) and WPI-glucose (●).

The levels of Schiff base increased with increasing DE value for all solutions containing carbohydrate on heating for 24 h at 90°C. The absorbance of the WPI-glucose solutions, in particular, increased dramatically compared to all other solutions. As glucose has the shortest carbonic chain length and, thus, considerably more free carbonyl groups per unit weight, this sample displayed the greatest formation of Schiff base. Similar results have been obtained by Guo and Xiong (2013) in heated systems containing buckwheat protein and maltodextrin.

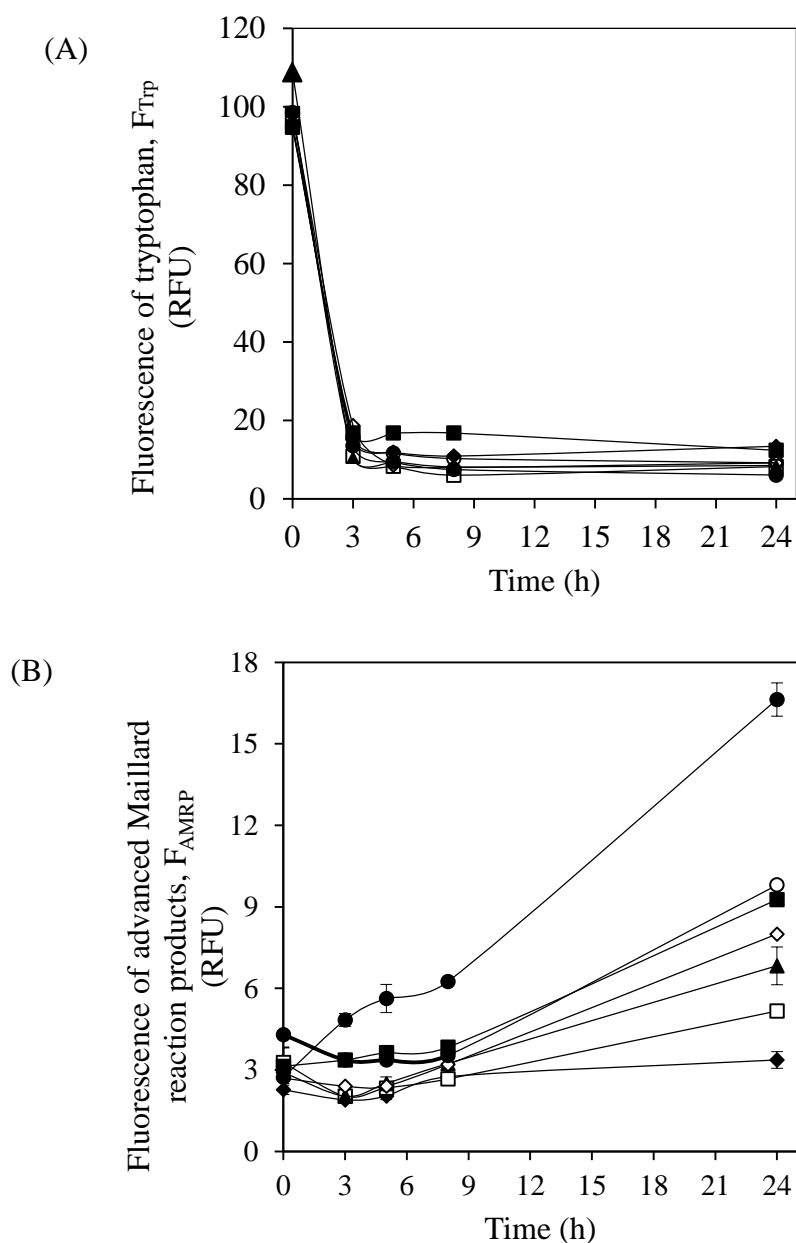
The presence of FUR in solutions has been reported to be a marker for the early stages of the Maillard reaction (Van Renterghem & De Block, 1996). The WPI and WPI-MD6/MD12/MD17 solutions heated for 8 h at 90°C did not have significantly ( $P > 0.05$ ) higher levels of FUR (13.6-24.2 mg 100 g<sup>-1</sup> protein, respectively) compared to the unheated WPI solution (4.1 mg 100 g<sup>-1</sup> protein). However, the level of FUR in the heated WPI-CSS30/CSS38 solutions (8 h at 90°C) was significantly ( $P \leq 0.05$ ) higher (366-371 mg 100 g<sup>-1</sup> protein) than the levels of FUR in the heated WPI and WPI-MD6/MD12/MD17 solutions. Van Renterghem and De Block (1996) reported similar levels of FUR (220-370 mg 100 g<sup>-1</sup> protein) in milk that was subjected to in-container sterilisation. The level of FUR in the heated WPI-glucose solution (73.7 mg 100 g<sup>-1</sup> protein) was lower than expected; while it may be counterintuitive to observe a lower FUR level in the heated solutions, this was attributed to the degradation of FUR into more advanced Maillard reaction products. Similar results were reported by Rufián-Henares *et al.* (2002) where the level of FUR decreased in whey-dominant model infant milk formulas on heating.



#### 3.4.4 Fluorescence of soluble tryptophan and advanced Maillard reaction products

Quantifying the changes in tryptophan fluorescence and concomitant accumulation of fluorescent Maillard products in the pH 4.6-soluble fraction allows an estimation of heat treatment and progression of the Maillard reaction into the advanced stages (Birlouez-Aragon *et al.*, 2002). The unheated WPI solution used in this study had relatively high soluble tryptophan fluorescence ( $F_{\text{Trp}}$ : 99.5 RFU, Fig. 3.4A). After 3 h of heating at 90°C, a significant ( $P \leq 0.05$ ) decrease in  $F_{\text{Trp}}$  was observed as the whey proteins underwent rapid denaturation and conformational changes; similar results have been reported by Birlouez-Aragon *et al.* (1998) and Da Silva Pinto *et al.* (2011). The formation of AMRP increased with heating time for all solutions, except the WPI control solution, i.e., with no added MD, CSS or glucose (Fig. 3.4B).

All WPI-MD/CSS solutions and the WPI control, had similar, low, levels of fluorescence due to AMRP (i.e., in the range 2.7-3.8 RFU) at 8 h of heating. The fluorescence of AMRP increased considerably between 8 and 24 h for all carbohydrate-containing solutions, as the Maillard reaction progressed into the advanced stages, in particular, for solutions containing CSS and glucose. Solutions containing WPI-CSS/glucose progressed into the later stages of the Maillard reaction faster than WPI-MD-containing solutions, as evidenced by a more rapid reduction in available amino groups and increase in fluorescence of AMRP in WPI-CSS30/CSS38/glucose samples. In order to maximise the conjugation of proteins and minimise the progression of the Maillard reaction into the later stages (with associated development of AMRP), 8 h of heating under the study conditions used herein was found to be optimal.



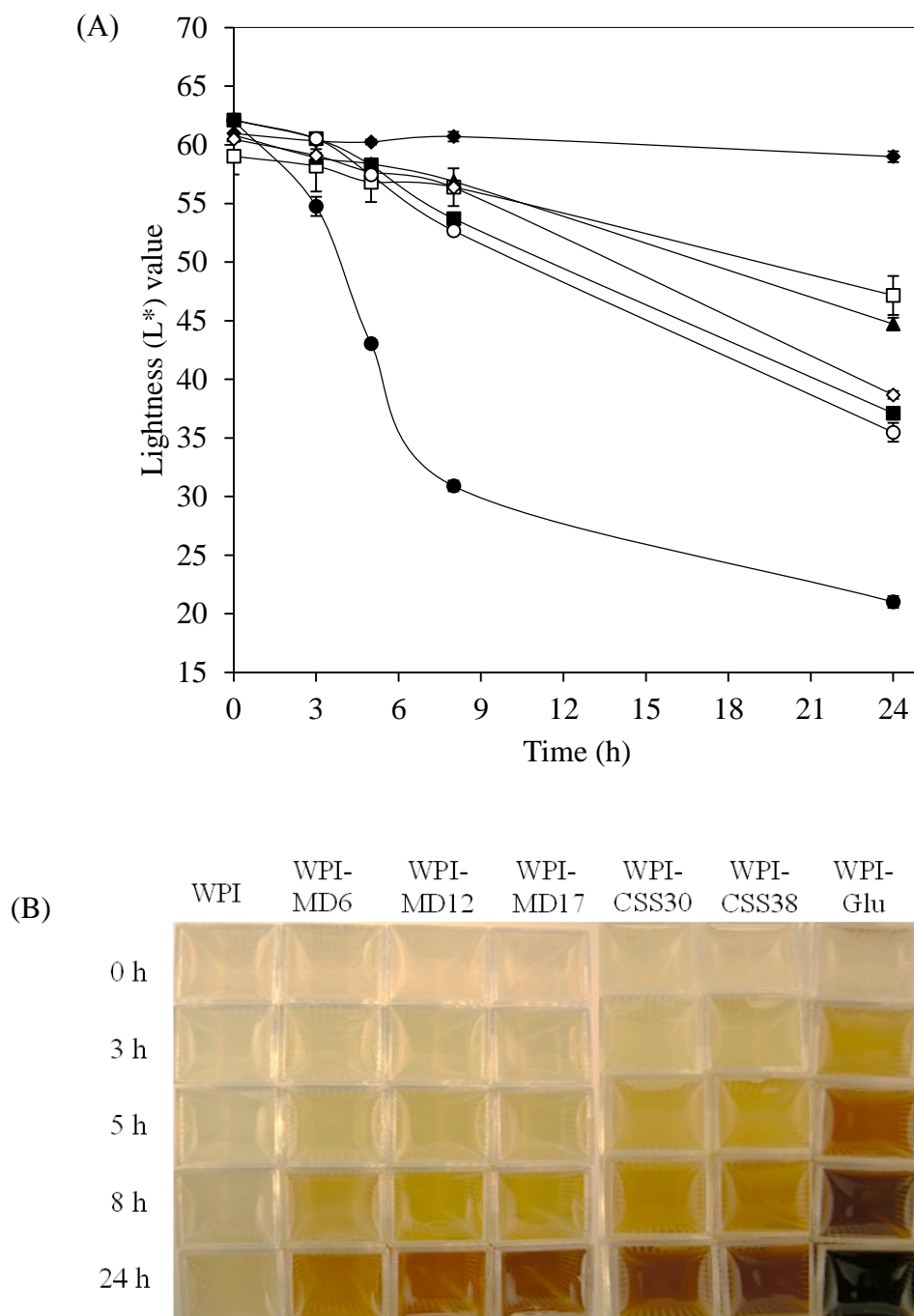
**Fig. 3.4.** (A) Fluorescence of tryptophan ( $F_{Trp}$ ) and (B) advanced Maillard reaction products ( $F_{AMRP}$ ), expressed in relative fluorescence units (RFU) in the pH 4.6-soluble fraction of whey protein isolate (WPI) and WPI-carbohydrate solutions containing maltodextrin (MD), corn syrup solids (CSS) or glucose of different dextrose equivalent values from 6 to 100 heated at an initial pH of 8.2 at 90°C for 24 h; WPI ( $\blacklozenge$ ), WPI-MD6 ( $\square$ ), WPI-MD12 ( $\blacktriangle$ ), WPI-MD17 ( $\diamond$ ), WPI-CSS30 ( $\blacksquare$ ), WPI-CSS38 ( $\circ$ ) and WPI-glucose ( $\bullet$ ).

#### 3.4.5 Colour development

Colourless products are formed in the initial condensation stage of the Maillard reaction, while yellow products are formed in the intermediate stage (Hodge, 1953). In the final stages, nitrogenous polymers and co-polymers, known as melanoidins, which are brown or yellow in colour are formed (Ames, 1998). At heating times  $> 8$  h, there were significant decreases ( $P \leq 0.05$ ) in the  $L^*$  value for all solutions containing starch hydrolysis products, with accompanying increases in yellow/brown colour (Figs. 2.5A and B). In particular, the WPI-glucose solution had greatest development of colour, as evidenced by the reduction in  $L^*$  value on heating for up to 24 h, indicating that the Maillard reaction progressed into the later stages faster and more extensively than for the WPI-MD/CSS containing solutions. The formation of AMRP associated with brown colour development is considered to be a negative aspect of conjugation (Poulsen *et al.*, 2013; Sithole *et al.*, 2005); thus, it is desirable to optimise conjugation between whey protein and starch hydrolysis products while limiting the development of AMRP and colour formation.

#### 3.4.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

In the reducing sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel stained for protein, two major bands corresponding to monomers of  $\beta$ -lg (18.4 kDa) and  $\alpha$ -lactalbumin ( $\alpha$ -lac, 14.2 kDa) were evident in unheated WPI (Fig. 3.6). On heating at 90°C for 8 h, the intensity of the  $\beta$ -lg and  $\alpha$ -lac monomer bands decreased considerably and large aggregates ( $>250$  kDa) that were not able to migrate into the gel formed in all heated samples. The DE value of the MD/CSS/glucose in the WPI-carbohydrate solutions influenced the extent of aggregation of the whey proteins

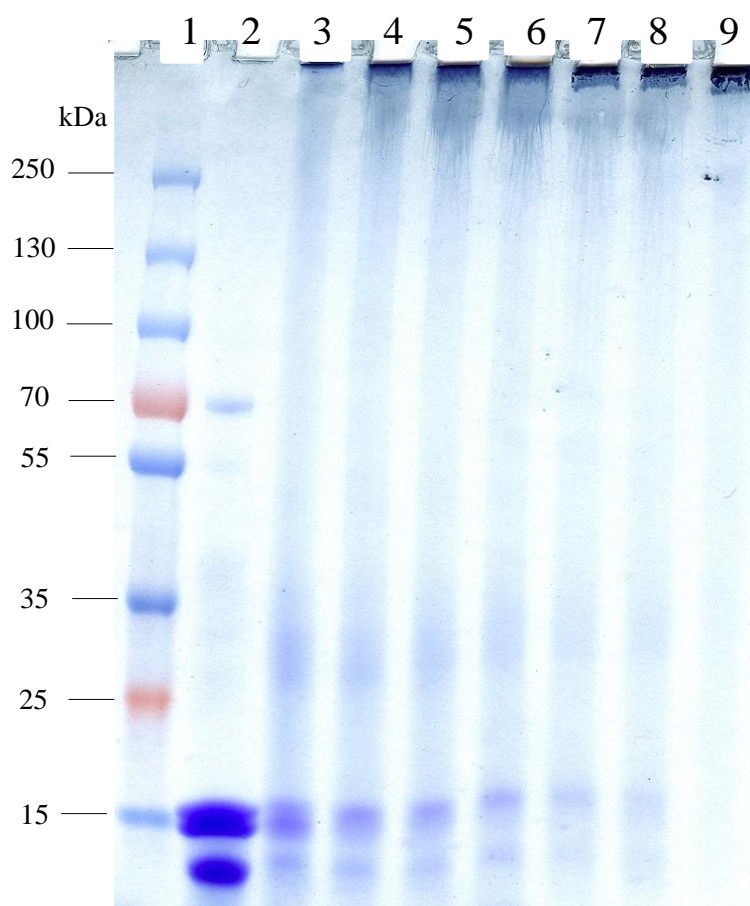


**Fig. 3.5.** (A) Colour chromaticity co-ordinate, expressed as  $L^*$  value and (B) photographic images of whey protein isolate (WPI) and WPI-carbohydrate solutions containing maltodextrin (MD), corn syrup solids (CSS) or glucose (Glu) of different dextrose equivalent values from 6 to 100 heated at an initial pH of 8.2 at 90°C for 24 h; WPI (◆), WPI-MD6 (□), WPI-MD12 (▲), WPI-MD17 (◇), WPI-CSS30 (■), WPI-CSS38 (○) and WPI-glucose (●).

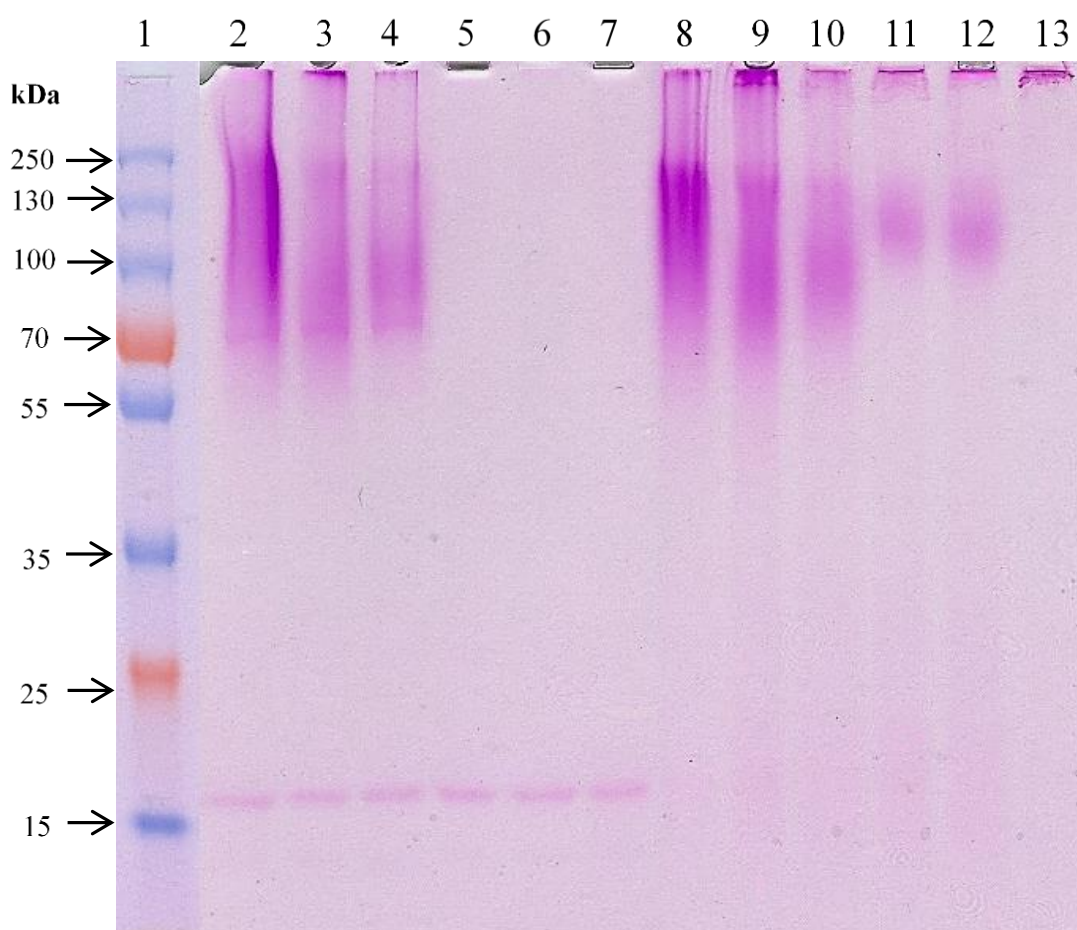
during heating, with less monomeric  $\beta$ -lg and  $\alpha$ -lac being evident on the gel with increasing DE value. In general, the bands corresponding to  $\beta$ -lg and  $\alpha$ -lac monomers at least partially disappeared on heating for all samples, with a distinct shift to a broad range of higher molecular weight species, indicating the role of covalent bonds other than disulphide bonding in aggregate formation at 8 h of heating. Heat treatment of protein, in combination with alkaline pH, can cause the formation of cross-linked amino acids such as lysinoalanine (Ames, 1998; Gerrard, 2002). Due to the extensive heating and exposure to moderate alkaline conditions used in this study, it is likely that covalent cross-linking of whey proteins resulted in the production of these large aggregates (Gerrard, 2002).

Fig. 3.7 shows the SDS-PAGE gel stained using the periodic acid-Schiff method to visualise the migration of carbohydrate conjugated to protein. The MD in the unheated WPI-MD solutions displayed limited migration into the gel; similar results were reported by O'Regan and Mulvihill (2009) where maltodextrin (DE value 4 and 10) migrated to a limited extent from the sample loading well into an SDS-PAGE gel. A possible explanation is that starch chains of sufficient length can bind with SDS forming starch-SDS inclusion complexes, which result in migration of the starch through SDS-PAGE gels, as shown by Debet and Gidley (2006). Due to its covalent attachment to whey proteins, the MD/CSS component associated with the conjugated protein migrated further and more extensively (compared with that for the unheated solutions) through the gel, particularly for heated WPI-CSS30 and WPI-CSS38 solutions. Similar results were reported by Dunlap and Côté (2005) and Wang and Ismail (2012) for dry heated  $\beta$ -lg-dextran and WPI-dextran systems. There was limited migration of the carbohydrate in the heated WPI-glucose solution which

was attributed to the sample not migrating through the gel due to the presence of large protein aggregates in the sample (Fig. 3.6, lane 9). The pink band at ~15 kDa is attributed to trace amounts of glycomacropeptide in the unheated WPI ingredient.



**Fig. 3.6.** One-dimensional sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoretograms stained for protein with Coomassie Brilliant Blue R-250. The lane identifications are as follows: (1) molecular weight marker, (2 and 3) WPI, (4) WPI-MD6, (5) WPI-MD12, (6) WPI-MD17, (7) WPI-CSS30, (8) WPI-CSS38, (9) WPI-glucose; lane 2 is unheated WPI and lanes 3-9 are WPI-carbohydrate solutions heated for 8 h at 90°C.



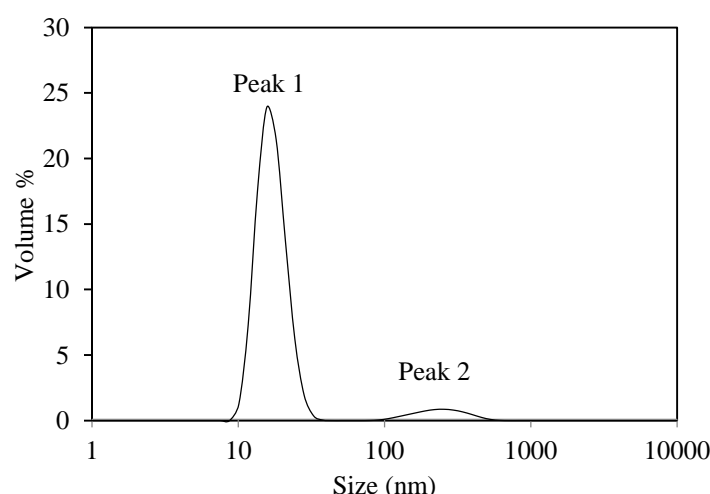
**Fig. 3.7.** One-dimensional sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoretogram stained for carbohydrate with periodic acid-Schiff reagent. The lane identifications are as follows: (1) molecular weight marker, (2 and 8) WPI-MD6, (3 and 9) WPI-MD12, (4 and 10) WPI-MD17, (5 and 11) WPI-CSS30, (6 and 12) WPI-CSS38, (7 and 13) WPI-glucose, lanes 2-7 are unheated solutions and lanes 8-13 are solutions heated for 8 h at 90°C.

#### 3.4.7 Particle size distribution

Heating WPI and WPI-carbohydrate solutions at 90°C for 8 h caused a shift in the volume-based size distribution profile of particles to a larger size compared to the respective unheated controls (Table 3.1). Both unheated and heated WPI and WPI-carbohydrate solutions displayed bimodal particle size distributions with two distinct peaks being identified (Fig. 3.8). In this study, the particles in the first peak (i.e., peak 1 corresponding to smaller size material) in unheated WPI solution had

volume-based mean diameters (VMD) of 17 nm which accounted for 93.8% of the total peak area, which increased to 36 nm at 8 h of heating at 90°C, indicative of aggregation of whey protein (Table 3.1). The second peak in unheated solution accounted for 6.2% of total peak area and had a VMD of 251 nm, which most likely resulted from low levels of denatured/aggregated protein in the WPI ingredient used. Similar results were obtained by Loveday *et al.* (2013) for unheated whey protein isolate and gum arabic solutions where 75% (v/v) of particles had a volume diameter between 20–30 nm and the remainder were 100–500 nm in diameter.

Heating of the WPI and WPI-carbohydrate solutions for 8 h at 90°C caused the formation of a small amount (<2.7% of total peak area) of relatively large particles with VMD of 2538-3998 nm. However, the heated WPI-glucose solution had smaller aggregates compared with WPI-MD/CSS solutions after heating; Da Silva Pinto *et al.* (2011) reported that the presence of glucose in  $\beta$ -lg solutions (initial pH 7, heated at 90°C for 24 h) prevented the formation of large covalently bonded-aggregates but did not impact the initial denaturation/aggregation step of native proteins.



**Fig. 3.8.** Identification of peaks in the particle size distributions by volume for the unheated WPI solution



**Table 3.1.** Particle size parameters of whey protein isolate (WPI) and WPI-carbohydrate solutions containing maltodextrin (MD), corn syrup solids (CSS) and glucose (glu) of different dextrose equivalent values from 6 to 100, unheated (0 h) or heated at an initial pH of 8.2 at 90°C for 24 h.

Sample	Heating Time	Total	Peak 1		Peak 2	
		Average Volume Diameter	Average Volume	Percent of	Average Volume Diameter	Percent of
			Diameter	Total Area		Total Area
	(h)	(nm)	(nm)	(%)	(nm)	(%)
WPI	0	32 ± 4	17 ± 1	93.8 ± 1.0	251 ± 9	6.2 ± 1.0
	8	97 ± 24	36 ± 2	97.7 ± 1.9	3,476 ± 399	2.3 ± 1.9
WPI-MD6	8	69 ± 23	32 ± 3	98.7 ± 1.0	3,697 ± 737	1.3 ± 1.0
WPI-MD12	8	53 ± 6	32 ± 2	99.4 ± 0.2	3,693 ± 245	0.6 ± 0.2
WPI-MD17	8	57 ± 10	35 ± 1	99.4 ± 0.2	3,998 ± 196	0.6 ± 0.2
WPI-CSS30	8	62 ± 11	34 ± 1	99.2 ± 0.5	3,814 ± 301	0.8 ± 0.5
WPI-CSS38	8	98 ± 14	36 ± 1	97.3 ± 0.8	2,538 ± 364	2.7 ± 0.8
WPI-Glu	8	39 ± 2	39 ± 2	100.0 ± 0.0	0 ± 0	0.0 ± 0.0

### 3.5 Conclusion

The dextrose equivalent value of starch hydrolysis products had a major impact on the rate of reduction of available amino groups during protein conjugation; the rate and extent of conjugation of whey proteins increased on heating for up to 8 h with increasing DE value of the starch hydrolysis products from MD (DE 6, 12 and 17), CSS (DE 30 and 38) to glucose. Heating of WPI-CSS38 for 8 h yielded the highest levels of conjugation with limited concomitant colour development compared with colour development in the solutions after 24 h of heating. All samples displayed increases in protein particle size on heating at 90°C, with the DE of the starch hydrolysis products influencing the size of particles formed; no large (>271 nm) particles formed in the whey protein-glucose solutions. Protein ingredients prepared by the conjugation of starch hydrolysis products to WPI have the potential to offer enhanced functionality in food applications and further investigation of the practical application of such conjugates in formulated food systems is required.

### 3.6 Acknowledgements

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## Chapter 4

### *Functional properties of whey protein conjugated with starch hydrolysis products of different dextrose equivalent values*

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## 4.1 Abstract

Conjugation of whey protein with maltodextrin (MD) or corn syrup solids (CSS) with dextrose equivalent (DE) values of 6 and 38 was achieved by heating solutions of 5% whey protein isolate (WPI) and 5% MD6 or CSS38, initial pH 8.2, at 90°C for 8 h. Conjugated WPI-MD6 and WPI-CSS38 solutions had lower levels of lysinoalanine (59.2 and 46.9 mg 100 g<sup>-1</sup> protein, respectively) than heated WPI (99.9 mg 100 g<sup>-1</sup> protein). The DE value of starch hydrolysis products affected the functionality of the conjugated protein. The apparent viscosity of WPI and WPI-MD6/CSS38 solutions increased after heating (pH 8.2, 90°C for 8 h) but overall, remained relatively low (<2.0 mPa.s). Conjugation increased whey protein solubility at pH 4.5 from 9% for whey protein heated alone to 24% for whey protein heated in the presence of CSS38 at 90°C for 8 h. Heating of all solutions reduced the ability of the material to generate a stable foam (over 60 min) compared to the respective unheated control solutions. Furthermore, conjugation of whey proteins with MD6 or CSS38 enhanced the stability to heating of protein solutions at 85°C for 3 min with 50 mM added NaCl.

## 4.2 Introduction

Whey proteins are widely used as food ingredients due to their excellent nutritional and functional properties. However, whey proteins can become unstable in high ionic strength environments during heating, which can result in aggregation and sedimentation of the protein (de Wit, 1998). Improvements in the functional properties of whey proteins at high salt concentration, high temperature, or in an acidic pH range is an on-going technological challenge.

Conjugation of proteins with carbohydrates through the Maillard reaction under controlled conditions has been shown to be effective in modifying protein functionality (Liu *et al.*, 2012; Nacka *et al.*, 1998; Oliver *et al.*, 2006; O'Regan and Mulvihill, 2010a,b). Several studies documenting the beneficial effects of protein modification through conjugation are available in the scientific literature, including improvements in solubility, emulsification, encapsulation and emulsion stability (Akhtar & Dickinson, 2003; Kasran *et al.*, 2013a, b), thermal stability (Jiménez-Castaño *et al.*, 2005; Kato *et al.*, 1995; Wang & Zhong, 2014) or foaming and gelation properties (Campbell *et al.*, 2003; Martínez & Pilosof, 2013). However, most of these studies report improvements in the functionality of protein-carbohydrate conjugates produced by a dry heating method, which is not currently feasible for large-scale production in the food industry (Zhu *et al.*, 2008, 2010). Zhu *et al.* (2010) studied the physicochemical and functional properties of whey protein isolate (WPI) conjugated with dextran in a heated aqueous environment (60°C for 24 h); however, no study has been reported on the functionality of whey protein-carbohydrate conjugate solutions produced using a shorter processing time at higher temperatures, which is economically desirable.

In this study, whey protein isolate (WPI), maltodextrin (MD) and corn syrup solids (CSS) were selected as the source of protein and carbohydrate, respectively. Previously, we reported that the dextrose equivalent (DE) value of starch hydrolysis products has a major impact on the rate of reduction of available amino groups during protein conjugation; heating of WPI-MD/CSS solutions for 8 h at 90°C yielded the highest levels of conjugation with limited concomitant colour development compared with the colour development in the solutions after 24 h of heating. The current study aims to investigate and report on the functionality (i.e., viscosity, protein solubility, thermal stability and foaming properties) of the heated WPI-MD/CSS conjugate solutions (8 h at 90°C) in comparison with the functionality of unheated WPI, WPI-MD/CSS and heated WPI control solutions.

### 4.3 Materials and Methods

#### 4.3.1 Materials

Whey protein isolate (WPI), BiPro<sup>®</sup> (with a chemical composition as outlined in Chapter 2) was obtained from Davisco Foods International (Le Sueur, MN, USA). Maltodextrin (MD) and corn syrup solids (CSS) with dextrose equivalent (DE) values of 6 (MD6) and 38 (CSS38), respectively, were obtained from Corcoran Chemicals Ltd. (Dublin, Ireland). All other chemicals were of analytical grade and were sourced from Sigma–Aldrich (Tallaght, Ireland).

#### 4.3.2 Preparation of WPI-carbohydrate conjugates

Blends of WPI (5%, w/v, protein) and MD6 or CSS38 (5%, w/v) were prepared in ultrapure water containing 0.02% sodium azide and allowed to solubilise for 2 h at 22°C with low speed magnetic stirring. The solutions were adjusted to pH 8.2 with 0.5 N KOH and allowed to hydrate for 18 h at 4°C, before being readjusted to pH 8.2 with 0.5 N KOH at 22°C. The conjugated protein solutions were prepared according to the method of Mulcahy *et al.* (2016a); briefly, aliquots (250 mL) of each solution were placed in 500 mL screw-capped, glass, conical flasks and heated at 90°C for 8 h in a pre-equilibrated, shaking water bath before being cooled immediately in iced water and, readjusted to pH 6.8 and stored at 4°C for further analysis. A control solution of WPI (5%, w/v, protein) was prepared, heated and sampled as described above.

#### 4.3.3 Determination of lysinoalanine

The level of lysinoalanine (LAL) in all solutions heated at an initial pH of 8.2 at 90°C for 8 h, and their respective unheated controls, was determined by reversed phase ultra-performance liquid chromatography (Acquity H Class, Waters Corporation, Milford, MA, USA). LAL was extracted as described by Calabrese *et al.* (2009) with minor modifications. Samples were not derivatised prior to analysis and the acid hydrolysate was diluted 1:10 with HPLC grade water. Injections (20 µL) were made onto a Waters Acquity HSS T3 C18 column (1.7 mm, 2.1 x 100 mm) at a flow rate of 0.5 mL min<sup>-1</sup> and maintained at 40°C. The isocratic mobile phase consisted of 0.1% formic acid in water. Detection was performed using a single quadrupole mass spectrometer (SQ Detector 2, Waters Corporation) in ES+ mode with the following settings: capillary voltage 0.35 V, sampling cone 38 V, source temperature 150°C, desolvation temperature 650°C, desolvation gas flow 1200 L h<sup>-1</sup> and LAL was quantified using the monovalent charged ion 234.04 in selective ion monitoring mode. An eight point standard curve was constructed ranging from 25 µg L<sup>-1</sup> to 5 mg L<sup>-1</sup> for quantification.

#### 4.3.4 Viscosity

The apparent viscosity of WPI, WPI-MD6 and WPI-CSS38 solutions heated at an initial pH of 8.2 at 90°C for 8 h and their respective unheated controls was determined based on the method of Mulcahy *et al.* (2016a) using a rotational viscometer (Haake RotoVisco 1 Rotational Viscometer, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a cylindrical double gap cup and rotor (DG43, Thermo Fisher Scientific). The shear rate was increased from 0.1 to 300 s<sup>-1</sup> over 5

min, under steady state conditions, held at  $300\text{ s}^{-1}$  for 2 min, and decreased from 300 to  $0.1\text{ s}^{-1}$  over 5 min. The average apparent viscosity at  $300\text{ s}^{-1}$  of each solution (2.5%, v/v, protein, pH 6.8) was determined at  $20\text{ }^{\circ}\text{C}$  ( $\pm 0.1^{\circ}\text{C}$ ).

#### 4.3.5 Protein Solubility

Protein solubility in the pH range 2–8 of WPI, WPI-MD6 and WPI-CSS38 solutions (0.1%, w/v, protein) heated at an initial pH of 8.2 at  $90\text{ }^{\circ}\text{C}$  for 8 h, and their respective unheated controls, was determined based on the method of O'Regan and Mulvihill (2009) with minor modifications. The pH of each solution was adjusted using 0.05 M HCl or NaOH before incubation at  $22\text{ }^{\circ}\text{C}$  for 1 h, after which the pH was checked and re-adjusted where appropriate. Each solution was then centrifuged at  $10,000\text{ g}$  for 20 min and filtered (Whatman No. 1) with the protein content of the supernatant and the respective initial solution being analysed using a BCA protein assay kit (Thermo Fisher Scientific). Results are reported as the protein content of each supernatant expressed as a percentage of the total protein content of the respective initial solution.

#### 4.3.6 Foaming

The foaming properties of WPI, WPI-MD6 and WPI-CSS38 solutions heated at an initial pH of 8.2 at  $90^{\circ}\text{C}$  for 8 h and their respective unheated controls was determined as described by Bartsch (1924) with minor modifications as detailed by Marinova *et al.* (2009). Solutions were adjusted to pH 6.8 with 0.05 N HCl at  $22^{\circ}\text{C}$  and diluted to 2.5%, w/v, protein with ultra-pure water prior to being transferred (25 mL) to a 120 mL glass cylinder (length, 250 mm; external diameter, 30 mm; wall



thickness, 3 mm). The glass cylinder was inverted 10 times and the initial foam volume, foam stability and liquid drainage for up to 60 min at 25°C were measured.

#### *4.3.7 Effect of heating at high temperature with added NaCl on turbidity development*

The development of turbidity in solutions of WPI and WPI-conjugates, with added salt, on heating at 85°C for 3 min was used as an index of thermal stability as described by Liu and Zhong (2012). As control samples, the transmission of light through unheated WPI, WPI-MD6 and WPI-CSS38 solutions (2.5 mL, 3.5%, w/v, protein, pH 6.8, 50 mM added NaCl) was measured at 400 nm using a Cary Bio UV-visible spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA). These solutions were heated at 85°C for 3 min in 10 mL glass tubes (length, 100 mm; external diameter, 12 mm; wall thickness, 2 mm) in a water bath before subsequent cooling in an ice bath (4°C) for 5 min before transmission was again measured. The solutions of WPI, WPI-MD6 and WPI-CSS38, previously heated at initial pH 8.2, for 8 h at 90°C (i.e., conditions required to achieve conjugation) were treated in the same manner to that described above. The transmission values of the respective control solutions and those heated at 85°C for 3 min are reported, along with the percentage reduction in transmission of those samples heated at 85°C for 3 min compared with the respective control.

#### *4.3.8 Statistical analysis*

The preparation of all solutions and their analysis was completed in three independent trials. Analysis of variance (one-way ANOVA) was carried out

followed by Duncan's mean comparison test to establish the significance in differences among the mean values using the Minitab 16 (Minitab Ltd, Coventry, UK, 2007) statistical analysis package and the level of significance was determined at  $P \leq 0.05$ .

## 4.4 Results and Discussion

### 4.4.1 Development of lysinoalanine on heating

The development of lysinoalanine (LAL) in protein solutions during heat treatment has been reported to be responsible for protein crosslinking leading to modification of functional properties and loss of nutritional value (Pellegrino *et al.*, 1999). The WPI solution heated for 8 h at 90°C had a significantly ( $P \leq 0.05$ ) higher level of LAL (99.9 mg 100 g<sup>-1</sup> protein) compared to the level in the unheated WPI solution (4.51 mg 100 g<sup>-1</sup> protein). However, the level of LAL in the heated WPI-MD6 and WPI-CSS38 solution (59.2 and 46.9 mg 100 g<sup>-1</sup> protein, respectively) at 8 h was significantly ( $P \leq 0.05$ ) lower than in the heated WPI solution. Heat treatment of proteins, especially at alkaline pH, can lead to the formation of LAL by  $\beta$ -elimination of seryl or cystyl residues to form the intermediate dehydroalanine, which can then react with the  $\epsilon$ -amino group of lysine (Calabrese *et al.*, 2009). As the  $\epsilon$ -amino groups of lysine are also consumed by the covalent attachment of MD to protein during the Maillard reaction, competition for the  $\epsilon$ -amino reaction sites is likely to be responsible for the lower levels of LAL found in heated WPI-MD/CSS solutions compared to the heated WPI solution.

#### 4.4.2 Change in viscosity on heating

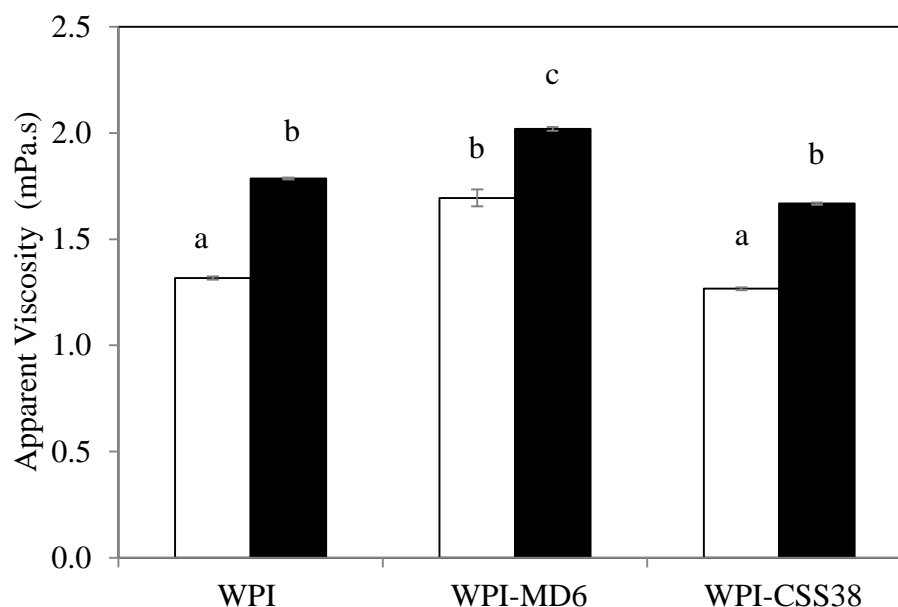
The apparent viscosity of the unheated WPI and WPI-MD6/CSS38 solutions was relatively low ( $<1.7$  mPa.s; Fig. 4.1). However, the heated WPI solution had a significantly ( $P \leq 0.05$ ) higher viscosity that was attributed to the presence of protein aggregates in the heated WPI solution, which have a larger effective volume compared to native whey protein molecules (Morr, 1989; Ryan *et al.*, 2012). The conjugated WPI-MD6 solution had a significantly ( $P \leq 0.05$ ) higher viscosity compared to the heated WPI solution; this was most likely due to the attachment of bulky maltodextrin molecules to the protein leading to an increase in the radius of gyration, which in turn, increased the effective volume occupied by the conjugated protein and carbohydrate molecules in solution (Hoffmann *et al.*, 1997; Kett *et al.*, 2013). Furthermore, hydration of MD molecules in the solution (i.e., unconjugated MD molecules) could have contributed to an increase in viscosity on heating potentially due to some unhydrolysed starch remaining in this ingredient.

There was no significant ( $P > 0.05$ ) difference between the apparent viscosity of the heated WPI solution (1.8 mPa.s) and the WPI-CSS38 conjugated solution (1.7 mPa.s). The attachment of corn syrup solids to protein molecules may not cause differences in the hydrodynamic radius of the conjugated WPI-CSS38 solution as the starch hydrolysis molecules in corn syrup solids have shorter carbonic chain lengths compared to maltodextrins.

#### 4.4.3 Protein Solubility

Unheated WPI and WPI-MD6/CSS38 solutions had high protein solubility ( $>87\%$ ) across the pH range 2–8 (Fig.4.2.) with minimum solubility occurring between pH

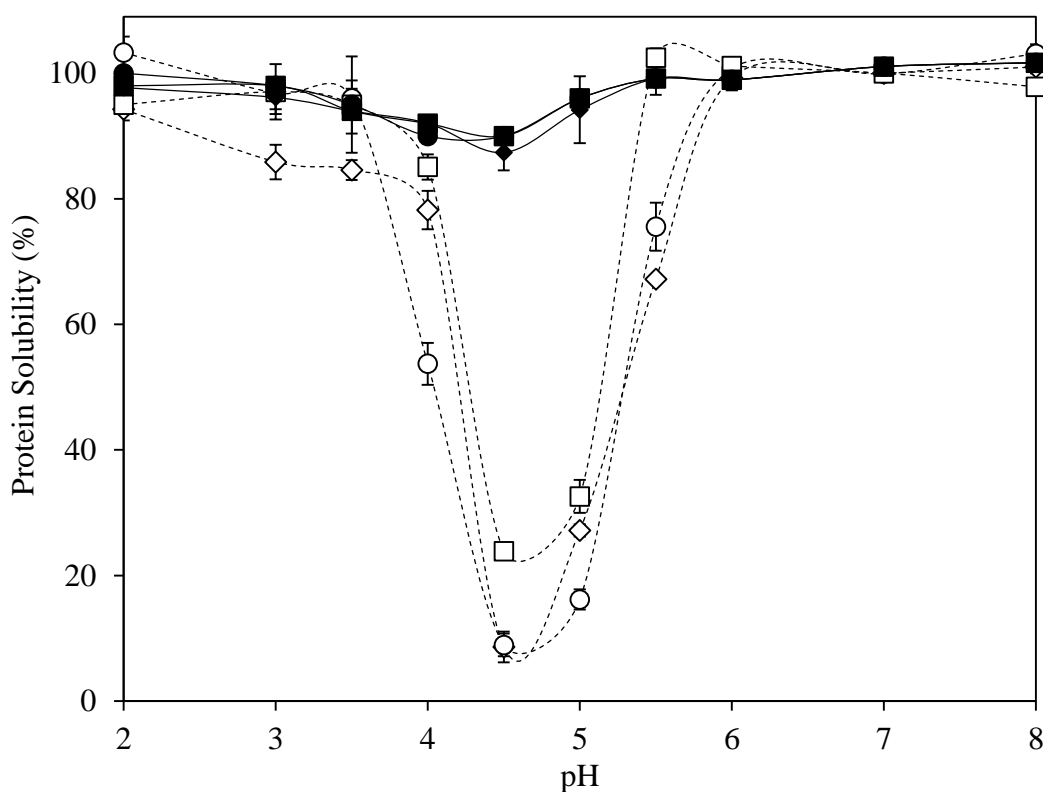
4.0 and 4.5. It is well established that the solubility of WPI is influenced by pH; near the isoelectric point of the protein, there is minimal intermolecular repulsion, and hence reduced protein solubility (de Wit, 1990).



**Fig. 4.1.** Apparent viscosity of whey protein isolate (WPI) and WPI-carbohydrate solutions (2.5%, v/v, protein) containing maltodextrin (MD6) and corn syrup solids (CSS38) either unheated (white) or heated (black) at an initial pH of 8.2 at 90 °C for 8 h. Different letters (i.e., a, b, c) on top of the bars denote that the total band volume values are significantly different from each other ( $P \leq 0.05$ ).

The protein solubility of the WPI solution previously heated at an initial pH of 8.2, at 90°C for 8 h was significantly lower ( $P \leq 0.05$ ) than that of the corresponding unheated WPI solution in the pH range 3.0–5.5, with the lowest protein solubility (9%) measured at pH 4.5 for the heated WPI solution, compared with a solubility of 87% for the unheated WPI solution. This decrease in solubility of whey protein on heat treatment is likely due to the increased levels of LAL and the thermal-induced unfolding of protein structure, exposing previously hidden reactive sites, resulting in greater protein-protein interactions and aggregation (Dissanayake *et al.*, 2013).

In contrast, all heated solutions exhibited high protein solubility (>97%) at pH 6.0–8.0, which was possibly attributable to increased charge-based repulsion, reducing protein-protein interactions (Morr & Ha, 1993). Solutions of conjugated WPI-MD6/CSS38, heated for 8 h, had higher protein solubility (>95%) in the pH range 2.0–3.5, compared with WPI solutions heated alone (80–90% protein solubility). In the pH range 4.0–5.5, the profile of the protein solubility curves for the WPI-MD6/CSS38 conjugate solutions were similar to that of the heated WPI solution, except that the WPI-CSS38 sample had significantly ( $P \leq 0.05$ ) higher protein solubility than the WPI-MD6 or heated WPI samples. Both the WPI-MD6



**Fig. 4.2.** Protein solubility as a function of pH of whey protein isolate (WPI) and WPI-carbohydrate solutions containing maltodextrin (MD6) or corn syrup solids (CSS38) unheated (—) or heated (---) at an initial pH of 8.2 at 90°C for 8 h; (WPI; ◆ or ◇) WPI-MD6 (● or ○) and WPI-CSS38 (■ or □).

These findings are in agreement with Wang and Ismail (2012), who reported improvements in solubility (8–9% increase) at pH 4.5–5.5 of WPI conjugated (initial pH 7, 49% relative humidity, dry heated at 60°C for 96 h) with dextran DE10 compared with unheated WPI. Wang and Ismail (2012) attributed this increase in solubility to reduced intermolecular protein interactions as the attachment of hydrophilic carbohydrate side chains (i.e., those from MD and CSS ingredients) to whey protein is likely to increase protein hydration and cause increased steric repulsion between proteins, resulting in improved solubility.

#### 4.3.4. Foaming Properties

No differences in the initial foam volume and subsequent foam decay were found between the unheated WPI and unheated WPI-MD6/CSS38 solutions or in liquid drainage from foams prepared from the unheated and heated WPI, WPI-MD6/CSS38 solutions (data not shown). The unheated WPI solution generated the greatest volume of foam (27.2 mL; Fig. 4.3) compared to the volume of foam produced from the heated WPI, WPI-MD6/CSS38 solutions (18.4–23.3 mL). Furthermore, the foam produced from the unheated WPI solution was very stable over 60 min of storage compared to the foam produced from the heated WPI, WPI-MD6/CSS38 solutions. Luck *et al.* (2002) and Norwood *et al.* (2016) reported that native whey proteins can produce stable foams as the flexibility of the whey protein molecules allow them to diffuse to the air-water interface, unfold and reorient to lower interfacial tension.

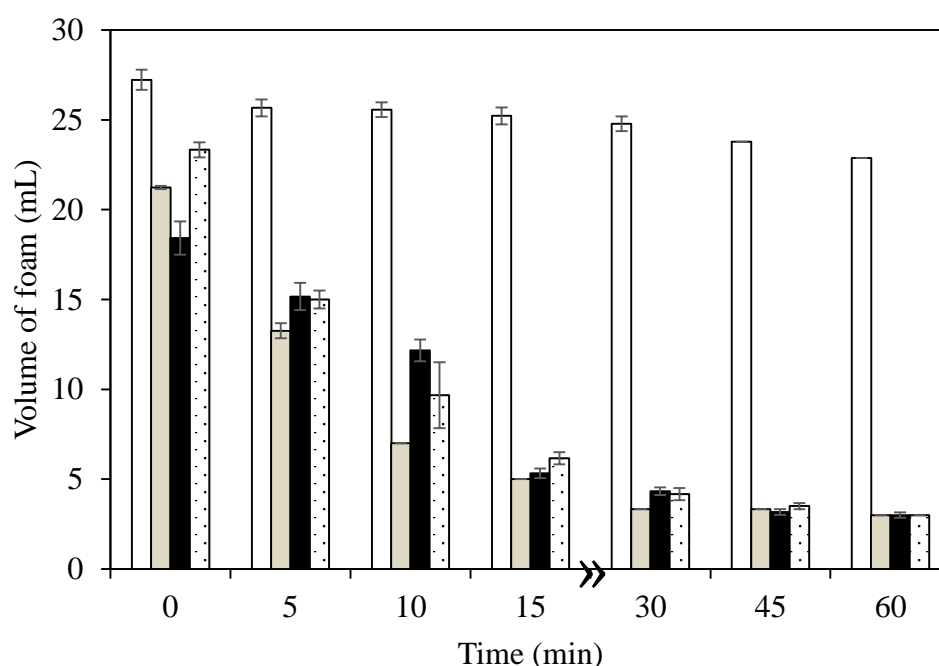
The heated WPI solution (8 h at 90°C) produced less foam (21.2 mL) which was very unstable over the 60 min storage period compared to the unheated WPI

solution; furthermore, coarsening of the foam was observed in the heated WPI sample, indicating that bubble coalescence and Ostwald ripening occurred (Fig. 4.3). Davis and Foegeding (2004) reported that heat-induced conformational changes (pH 7, 10% w/v protein, 80°C for 30 min) in the structure of the whey protein molecules can confer improved foaming properties as they whey proteins become more flexible and coadsorbed with native whey proteins, thus allowing more rapid formation of the air/water interfacial layer.

Zhu and Damodaran (1994) reported that foams produced from heated WPI solutions (90°C for 90 min) were less stable than those formed from unheated WPI solutions due to the presence of large whey protein aggregates which limited the migration of the protein molecules to the air-solution interface. Moreover, Kamath *et al.* (2008) reported that the foaming properties of heat-treated whey protein solution can be impaired due to protein aggregation, which decreases the ability of proteins to form a cohesive proteinaceous layer at the air–water interface (i.e., hindering foam stability).

The conjugated solution of WPI-MD6 (heated at 90°C for 8 h) generated the least volume of foam (18.3 mL) relative to the unheated WPI and heated WPI and WPI-CSS38 solutions. Báez *et al.* (2013) reported that less foam was produced from dry heated  $\beta$ -lactoglobulin ( $\beta$ -lg)-glucose (96 h at 50°C) compared to unheated  $\beta$ -lg, due to the presence of large protein aggregates which led to a disruption in the viscoelasticity of the interfacial film. However, the WPI-MD6 solution displayed slightly better foam stability for up to 10 min of storage compared to the heated WPI and WPI-CSS38 solutions. This slight improvement in foam stability may also be attributed to the higher viscosity of the heated WPI-MD6 solution (Fig. 4.1) which

may have caused a more cohesive viscoelastic film to form between adjacent air bubbles, limiting liquid drainage. These results are in agreement with Corzo-Martínez *et al.* (2012) who reported that foam produced from conjugated  $\beta$ -lg-galactose (dry heated at 50°C for 48 h) was more stable than foam produced from dry heated  $\beta$ -lactoglobulin (without galactose) due to increased molecular flexibility and higher diffusion coefficients of the conjugated  $\beta$ -lactoglobulin-galactose which improved foam stability. The conjugated WPI-CSS38 solution had a similar foam stability profile to the conjugated WPI-MD6 solution except that more foam was initially generated in the conjugated WPI-CSS38 solution (23.3 mL) than in the conjugated WPI-MD6 solution (18.3 mL).



**Fig. 4.3** Volume of foam generated from 10 inversions of 25 mL of solution in a closed glass cylinder and subsequent decay of foam on storage for up to 60 min of whey protein isolate (WPI) and conjugated WPI-maltodextrin (MD6) or -corn syrup solids (CSS38) solutions at pH 6.8 and 2.5% protein. Identifications are as follows; unheated WPI (white bar), heated WPI (light grey bar), heated WPI-MD6 (black bar) and heated WPI-CSS38 (dotted bar); heated solutions were treated for 8 h at 90°C.



Larger protein aggregates were present in the conjugated WPI-MD6 solution compared to the conjugated WPI-CSS38 solution, which is likely to have caused a steric impediment in the migration of the protein molecules to the air-solution interface.

#### *4.4.5. Effect of heating at high temperature with added NaCl on turbidity development*

Previously unheated WPI and WPI-MD6/CSS38 solutions with 50 mM added NaCl showed considerable increases in turbidity (43.6–50.7% reduction in transmission of light compared with unheated controls) when heated at 85°C for 3 min, due to the formation of protein aggregates that scattered light (Table 3.1; Fig. 4.4). The solution of WPI that had been heated for 8 h at 90°C before receiving a further heat treatment of 3 min at 85°C with 50 mM added NaCl displayed a 33.3% reduction in transmission compared with the respective control. These results are in keeping with those of Sağlam *et al.* (2014) who reported that pre-aggregated (formed through emulsification of a 25% WPI solution with sunflower oil and heating at 80°C for 20 min at pH 5.5) whey protein were less susceptible to heat-induced structural changes and gelation on further heating at 90°C, pH 3.6 for 30 min, than native whey protein.

Solutions of WPI-MD6 and WPI-CSS38 conjugates (i.e., previously heated for 8 h at 90°C at an initial of pH 8.2) displayed a reduction in transmission of just 17.9 and 12.5%, respectively, after heating for 3 min at 85°C with 50 mM added NaCl (Fig. 4.4). The lower values for transmission of light through the conjugated solutions of WPI-MD6/CSS38 (39.0 and 7.2 relative transmission units)

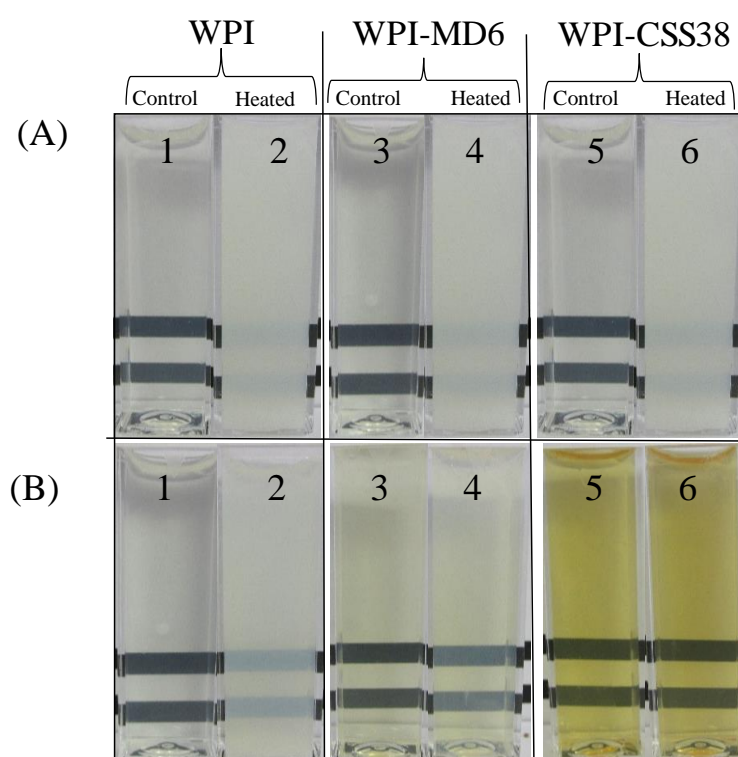
**Table 4.1.** Development of turbidity as measured by transmission of light at 400 nm through whey protein isolate (WPI) and WPI-carbohydrate solutions containing maltodextrin or corn syrup solids previously unheated (0 h) or heated at 90°C for 8 h at an initial pH of 8.2.<sup>a</sup>

Solution	Treatment	Transmission of light			
		0 h	% reduction	8 h	% reduction
	*	RTU (-)	(-)	RTU (-)	(-)
WPI	C <sup>a</sup>	71 ± 0.5	na	69 ± 0.3	na
	H <sup>a</sup>	40 ± 2.1	43.6	46 ± 7.4	33.3
WPI-MD6 <sup>a</sup>	C	71 ± 0.5	na	39 ± 0.0	na
	H	35 ± 3.8	50.7	32 ± 0.3	17.9
WPI-CSS38 <sup>a</sup>	C	71 ± 0.5	na	7.2 ± 0.0	na
	H	39 ± 1.0	45.1	6.3 ± 0.0	12.5

<sup>a</sup>Abbreviations are: MD, maltodextrin; CSS, corn syrup solids; RTU, relative transmission units; C, control (solutions diluted to 3.5% protein, adjusted to pH 6.8 and 50 mM NaCl added); H, as for control, but further heated for 3 min at 85°C; na, non-applicable.

relative to the heated WPI solution (69.0 relative transmission units) is attributed to the absorbance of colour at 400 nm rather than the development of turbidity in the solutions. It is clear from these results that covalent attachment of the MD and CSS to whey protein molecules through Maillard-induced conjugation led to considerably greater stability and clarity of whey protein solutions when thermally processed. The covalent attachment of carbohydrate side chains had a strong additive effect to that of heat-induced aggregation/stabilisation of the whey proteins under the heat treatment conditions (i.e., 8 h at 90°C) required to achieve conjugation. The improvement in thermal stability of whey protein following conjugation with MD or CSS is most likely due to enhanced steric hindrance due to the attachment of

carbohydrate side chains to whey protein molecule helping to prevent further heat-induced protein aggregation, which can cause increased turbidity. These results are in keeping with those of Liu and Zhong (2012) who reported enhanced stability to heating (88°C for 2 min in the pH range 3.0–7.0, with 0–150 mM added NaCl or CaCl<sub>2</sub>) of whey protein (7.0%) when conjugated with maltodextrin (DE of 18).



**Fig. 4.4.** Photographs of whey protein isolate (WPI) and WPI-carbohydrate solutions containing maltodextrin with a dextrose equivalent value of 6, (MD6) or corn syrup solids with a dextrose equivalent value of 38 (CSS38); WPI (1, 2), WPI-MD6 (3, 4) and WPI-CSS38 (5, 6) solutions, pH 6.8, containing 3.5% protein and 50 mM added NaCl (control), and after heating at 85°C for 3 min (heated). Photographs of samples labelled with (A) received no prior heat treatment (unconjugated) and (B) were previously heated at 90°C for 8 h at an initial pH of 8.2 (to induce conjugation).

## 4.5 Conclusion

This study showed that the functionality of WPI can be markedly improved through Maillard reaction-induced conjugation with starch hydrolysis products (i.e., MD or CSS). Heating (8 h at 90°C) of the WPI-MD6/CSS38 solutions to induce conjugation was achieved with a relatively small concurrent increase in apparent viscosity. The level of lysinoalanine in the conjugated WPI-MD6/CSS38 solution was significantly ( $P \leq 0.05$ ) lower than that in the heated WPI solution due to competition for the reactive  $\epsilon$ -amino group of lysine. Solutions of conjugated WPI-CSS38 displayed increases in protein solubility of 6-32% at pH 4-5.5 compared with solutions of conjugated WPI-MD6 and heated WPI solutions, due to increased steric repulsion between the protein molecules. Furthermore, conjugation of WPI with CSS38 for 8 h maintained high solution clarity on heating with 50 mM added NaCl compared with conjugated WPI-MD6, unheated WPI-MD6 and WPI solutions. Conjugation of WPI-MD6/CSS38 solutions also resulted in improvements in the foam stability up to 10 min of storage compared to the heated WPI solution. Protein ingredients prepared by the conjugation of starch hydrolysis products to WPI have the potential to offer enhanced functionality in food applications and further investigation of the practical application of such conjugates in formulated food systems is required.

## 4.6 Acknowledgements

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## Chapter 5

### *Enhancement of the functional properties of whey protein by conjugation with maltodextrin under dry heating conditions*

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## 5.1 Abstract

Conjugation of whey proteins in whey protein isolate (WPI) and maltodextrin (MD) with a dextrose equivalent value of 6 (MD6) was achieved by heating at an initial pH of 7.0, at 60°C and 79% relative humidity, in the ratio WPI:MD6 of 1:1, for up to 24 h. The level of available amino groups in the WPI-MD6 sample decreased significantly ( $P \leq 0.05$ ) by 10.4% (compared to the unheated WPI-MD6 control) on heating for 24 h. Limited colour developed and the production of advanced Maillard products was low in the WPI-MD6 sample after 24 h of heating. Conjugation of WPI with MD6 increased the protein solubility near the isoelectric point (i.e., at pH 4.5) by 7.1-8.4% compared to the unheated WPI and heated WPI controls. Conjugation of WPI with MD6 enhanced the stability and clarity of protein solutions, compared with WPI and heated WPI controls, when heated at 85°C for 10 min with 50 mM added NaCl.

## 5.2 Introduction

Whey protein ingredients are utilised in the formulation of a wide range of food and clinical nutritional products, due to their nutritional and functional attributes including solubility, gelation, aeration, water-binding and emulsification properties (Smithers, 2015). In particular, the nutritional value of whey proteins has contributed strongly to their use in a variety of high protein beverages (Foegeding *et al.*, 2002). The food and drug administration requires that a minimum of 20% of the energy value of a beverage is provided by protein to justify a claim of “high protein” (FDA, 21 CFR 101.54 B), depending on other constituents and total solids content. Thermal processing of nutritional beverages is required to ensure microbiological safety but the inherent instability of whey proteins to heating, due to protein denaturation and aggregation, can often limit the concentration of whey protein in beverages. Aggregation of whey proteins can lead to diminished processing stability and subsequent deterioration of product quality during storage, including increased viscosity and gelation, or excessive turbidity, manifesting, under extreme conditions, as phase separation or precipitation (LaClair & Etzel, 2010).

Conjugation of proteins with reducing carbohydrates occurs during the early stages of the Maillard reaction when the  $\alpha$ -amino group of lysine, or the  $\epsilon$ -amino groups of terminal amino acids, condense with the carbonyl groups of reducing carbohydrates (Ames, 1992). Protein-carbohydrate conjugation *via* the Maillard reaction has been shown to be an effective method to modify the techno-functionality of proteins, including improving protein solubility and thermal stability (Chevalier *et al.*, 2001; Liu *et al.*, 2012; Mulcahy *et al.*, 2016a,b; Chapters 3 and 5). Some previous studies focused on improvement in functionality (e.g., protein solubility

and thermal stability) of individual whey protein fractions (i.e.,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, bovine serum albumin) conjugated with carbohydrates by dry heating for several days (Jiménez-Castaño *et al.*, 2007). Wang and Ismail (2012) reported enhanced protein solubility (at pH 4.5-5.5) and thermal stability (at 85°C) of whey protein isolate (WPI) conjugated with dextran at 60°C and 49% relative humidity, for 96 h. Liu and Zhong (2012) reported minor improvements in the thermal stability of WPI dry heated at an initial pH of 7.0, at 60°C and 80% relative humidity (RH) for 24 h with maltodextrin (dextrose equivalent of 18) but did not quantify the levels of advanced Maillard reaction products in the system.

A previous study by the authors (Mulcahy *et al.*, 2016a, Chapter 2) reported that dextrose equivalent value of starch hydrolysis products (i.e., maltodextrin or corn syrup solids) impacted the functionality of a conjugated whey protein-carbohydrate system. The present study focused on a systematic evaluation of the influence of limited dry heating (up to 24 h) on the rate and extent of conjugation of whey proteins with maltodextrin of a dextrose equivalent of 6, which was chosen in order to maximise the alteration in the techno-functional properties (i.e., protein solubility and thermal stability). A reaction time course of up to 24 h was used to monitor the progression of the Maillard reaction and to characterise Maillard reaction products formed at the early and advanced stages.

## 5.3 Materials and Methods

### 5.3.1 Materials

Whey protein isolate (WPI), BiPro<sup>®</sup>, containing 89.4% protein, 4.5% moisture, <0.5% fat, 3.0% ash and 0.03% lactose (measured as described by Mulcahy *et al.*, 2016a, Chapter 2) was obtained from Davisco Foods International (Le Sueur, MN, USA). Maltodextrin with a dextrose equivalent value of 6 (MD6) containing 4.1% moisture and 0.1% ash was obtained from Corcoran Chemicals Ltd, Dublin, Ireland. All solutions were prepared with ultra-pure water. All other chemicals were of analytical grade and were sourced from Sigma–Aldrich (Dublin, Ireland), unless stated otherwise.

### 5.3.2 Preparation of dry heated WPI-maltodextrin conjugates

Dry heated conjugates of WPI-MD6 were prepared using a modification of the method previously described by O'Regan and Mulvihill (2009). Solutions were prepared as follows; a mixture of WPI (5%, w/v, protein) and MD6 (5%, w/v, carbohydrate) was reconstituted in ultrapure water containing 0.02% sodium azide and allowed to hydrate for 2 h at 22°C with low speed magnetic stirring. The solution was adjusted to pH 7.0 using 0.1 M HCl or 0.1 M NaOH, stored at 4°C for 18 h and re-adjusted to pH 7.0, as necessary. The WPI-MD6 solution was frozen initially at –20°C for 12 h and then tempered at –80°C for 3 h. Freeze-drying was carried out under vacuum at a pressure of <0.1 mbar for 72 h. The resulting material was milled, transferred to a plastic petri dish (150 mm × 15 mm) and then placed on a perforated plate in a desiccator that contained a pre-equilibrated saturated potassium bromide solution generating an atmosphere with a relative humidity (RH) of 79% at 60°C.

The freeze-dried material was dry heated for 24 h at 60°C and samples were removed after 3, 5, 8 and 24 h, after which the material was freeze-dried again to remove excess moisture. A control sample containing WPI alone was prepared, heated and sampled as described above.

### 5.3.3 Determination of available amino groups

The change in the level of available amino groups in solutions (0.1%, w/v, protein) prepared from dry heated WPI and WPI-MD6 was measured by the o-phthalaldehyde (OPA) method as detailed by Nielsen *et al.* (2001) with minor modifications as described by Mulcahy *et al.* (2016a). The concentration of available amino groups in the WPI and WPI-MD6 solutions at 3, 5, 8 and 24 h of heating was expressed as a percentage of available amino groups in the respective unheated solutions.

### 5.3.4 Determination of furosine and lysinoalanine

The concentration of furosine (FUR) in solutions (5%, w/v, protein) prepared from the dry heated WPI and WPI-MD6 after 24 h of heating, and their respective unheated controls, was quantified by ion-pair reversed-phase high-performance liquid chromatography. FUR was extracted as described in ISO (2004) and 0.5 mL was passed through a 500 mg solid phase extraction column (Discovery DSC-18, Supelco), previously conditioned with 5 mL of methanol and 10 mL of ultra-pure water, and the FUR was eluted with 3 mL of 3 M HCl. Injections (20  $\mu$ L) were made onto a dedicated FUR column (Luna C8, 5  $\mu$ m, 4.6 x 250 mm, 100Å; Phenomenex, Torrance, CA, USA) at a flow rate of 0.5 mL min<sup>-1</sup> which was maintained at 35°C.



The isocratic mobile phase used was 0.4% acetic acid in ultra-pure water. Detection was performed at 280 nm using a photodiode array detector (model 2998, Waters Corporation). An eight point standard curve ranging from 50 to 400 mg mL<sup>-1</sup> FUR was used for quantification.

The concentration of lysinoalanine (LAL) in solutions prepared from the dry heated WPI and WPI-MD6 samples after 24 h of heating, and their respective unheated controls, was quantified by reversed phase ultra-performance liquid chromatography as described by Mulcahy *et al.* (2016b).

#### 5.3.5 Fluorescence of advanced Maillard reaction products

The fluorescence of advanced Maillard reaction products (F<sub>AMRP</sub>) were measured as described by Mulcahy *et al.* (2016b). The fluorescence of the pH 4.6 soluble fraction of solutions were measured at excitation/emission wavelengths of 330/420 nm for advanced Maillard reaction products (F<sub>AMRP</sub>) and expressed in relative fluorescence units per gram of protein (RFU g<sup>-1</sup> protein).

#### 5.3.6 Measurement of colour

The colour of solutions (5%, w/v, protein), prepared from dry heated WPI and WPI-MD6, was determined using a pre-calibrated colorimeter (Minolta Chroma Meter CR-400, Minolta Ltd., Milton Keynes, UK) as described by Mulcahy *et al.* (2016a). Colour was expressed in the form of Commission Internationale de l'Eclairage (CIE) L\* a\* b\* colour chromaticity coordinates.

### 5.3.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of dry heated WPI and WPI-MD6 (24 h), and their respective unheated controls, was performed under reducing and non-reducing conditions according to the method of Laemmli (1970) with minor modifications to protein and carbohydrate staining protocols as detailed by Mulcahy *et al.* (2016a).

### 5.3.8 Level of whey protein denaturation as determined by pH 4.6-soluble protein

The level of pH 4.6-soluble protein in solutions (5%, w/v, protein), prepared from the dry heated WPI and WPI-MD6 after 24 h of heating, and their respective unheated controls, was quantified as described by O'Kennedy and Mounsey (2006), with the following minor modifications. Acetic acid (10%, v/v; 4 mL) was added to each solution (50 mL), which was then incubated at 40°C for 10 min, before 4 mL of sodium acetate (1 M) was added. Each solution was then further incubated at 40°C for 10 min and allowed to cool to 22°C. The pH was readjusted to pH 4.6 with either 10% acetic acid or 1 M sodium acetate (as required), and the solutions were centrifuged at 10,000 g at 22°C for 20 min. The nitrogen content of the supernatant was determined by the Kjeldahl method (AOAC, 1995). Results are reported as the pH 4.6 soluble protein (i.e., native protein) expressed as a percentage of the total protein in the solution.

### 5.3.9 Particle size distribution

The size of particles in the solutions (0.1%, w/v, protein), prepared from dry heated WPI and WPI-MD6 (60°C and 79% RH for 24 h), was measured by dynamic light scattering (DLS) with a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) as described by Mulcahy *et al.* (2016a). The overall volume-based mean diameter (VMD) of the unheated and heated WPI and WPI-MD6 solutions is reported along with the percentage area and VMD of individual peaks in the particle size distribution profile.

### 5.3.10 Transmission electron microscopy

Negative staining of solutions (0.05%, w/v, protein), prepared from dry heated WPI and WPI-MD6, was completed essentially as described by Loveday *et al.* (2010), except that the solutions were not centrifuged or filtered before being stained with 2% uranyl acetate (diluted in ultra-pure water). Specimens were imaged using a JEOL Transmission Electron Microscope JEM 2000FXII (Jeol Ltd., Tokyo, Japan), operated at 80 kV. Electron micrographs were obtained using a Megaview-III digital camera (EMSIS GmbH, Mendelstrasse, Muenster, Germany) and AnalySIS software. At least three specimens of each sample were observed and representative micrographs are presented.

### 5.3.11 Viscosity

The apparent viscosity of solutions (2.5%, w/v, protein), prepared from the dry heated WPI and WPI-MD6 (heated at 60°C and 79% RH for 24 h), and their

respective unheated controls, was measured by rotational viscometry using the method described by Mulcahy *et al.* (2016a).

#### 5.3.12 Protein Solubility

Protein solubility of solutions (0.1%, w/v, protein), prepared from dry heated WPI and WPI-MD6 (heated at 60°C and 79% RH for 24 h), and their respective unheated controls, was determined in the pH range 2-8 using the method described by Mulcahy *et al.* (2016a).

#### 5.3.13 Effect of heating in the presence of added salt on turbidity development

The development of turbidity in the presence of added salt, when solutions prepared from the dry heated powders were further heated, and their respective unheated controls, was determined based on the method of Liu and Zhong (2012). WPI and WPI-MD6 powders that were previously dry heated for 24 h (i.e., to achieve conjugation) were used to prepare solutions containing 5.0%, w/v, protein, 50 mM added NaCl was then added and the pH was adjusted to 6.8 before 2.5 mL was heated at 85°C for 10 min in 10 mL glass tubes (length, 100 mm; external diameter, 12 mm; wall thickness, 2 mm) in a water bath before the tubes were immediately cooled by immersion in iced water for 5 min. Unheated WPI and WPI-MD6 powders (i.e., that were not previously dry heated), were used to prepare control solutions containing 5.0%, w/v, protein, 50 mM added NaCl, pH 6.8 (2.5 mL), that were subsequently heated at 85°C for 10 min as described above. The transmission of light through all solutions was measured at 400 nm using a Cary 300 Bio UV-visible spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA). The

percentage reduction in transmission of light through the solutions heated at 85°C for 10 min, compared to the respective control is reported.

#### 5.3.14 Statistical data analysis

The preparation of all solutions and their analysis was completed in three independent trials. Analysis of variance (ANOVA) was carried out, followed by Tukey's mean comparison test, to establish the significance of differences among the mean values using the Minitab 16 (Minitab Ltd, Coventry, UK, 2007) statistical analysis package and the level of significance was determined at  $P \leq 0.05$ .

## 5.4 Results and Discussion

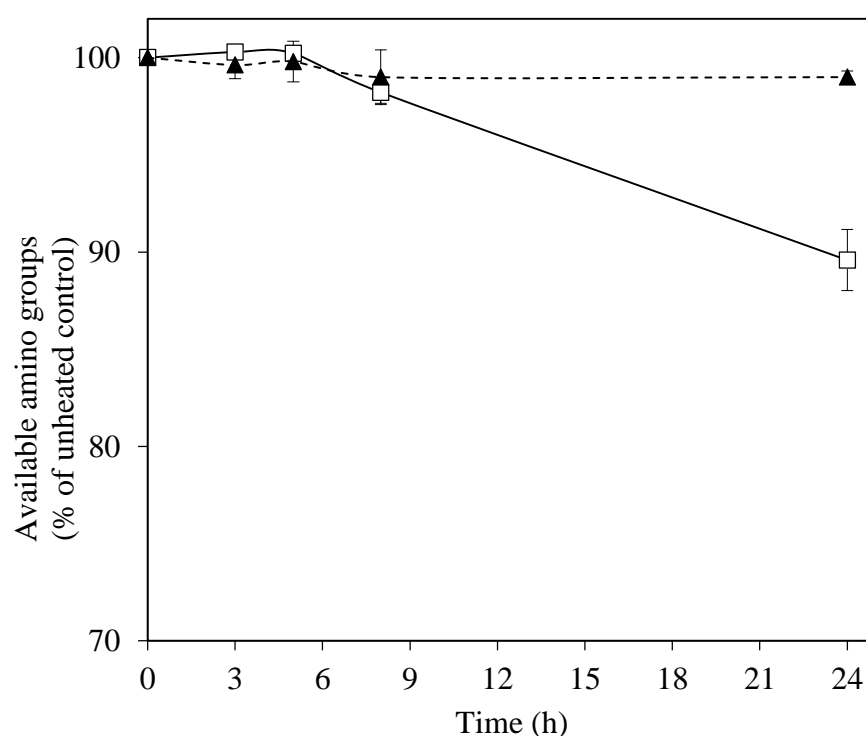
### 5.4.1 Available amino groups

The level of available amino groups (AAG) in the WPI control decreased by 0.9% on dry heating for 24 h (Fig. 5.1). This slight reduction may be attributed to some structural changes within the protein molecules and/or Maillard-induced reactions of the proteins with the innate reducing carbohydrate, lactose, present in the WPI resulting in blockage of the AAG. Chevalier *et al.* (2001) reported similar results in a heated (at 60°C, at pH 6.5 for 72 h)  $\beta$ -lg solution where a 6.2% reduction in AAG was recorded. There was no significant ( $P > 0.05$ ) decrease in the level of AAG in WPI-MD6 up to 8 h of dry heating; after a further 16 h of dry heating, the level of AAG in the WPI-MD6 was significantly ( $P \leq 0.05$ ) lower than the level of AAG in the respective unheated WPI-MD6 control (Fig. 5.1). Therefore, the WPI-MD6 conjugate prepared by dry heating for 24 h was chosen for further assessment

of its physicochemical and functional properties (i.e., viscosity, protein solubility and thermal stability).

#### 5.4.2 Development of furosine and lysinoalanine on heating

The presence of furosine (FUR) is reported to be a marker for the early stages of the Maillard reaction as it is derived from Amadori compounds produced from the reaction of AAG with reducing carbohydrates (Erbersdobler & Somoza, 2007). There were no significant ( $P > 0.05$ ) differences in the concentration of FUR in unheated WPI and unheated WPI-MD6 (Table 4.1).



*Fig. 5.1. Level of available amino groups in solutions prepared from whey protein isolate (WPI; ▲) and WPI-maltodextrin (WPI-MD6; □) dry heated at an initial pH of 7.0 at 60°C and 79% relative humidity for up to 24 h. Values are presented as mean  $\pm$  standard deviation of data from three independent trials.*

Dry heating of WPI alone for 24 h resulted in a significantly ( $P \leq 0.05$ ) higher concentration of FUR than in the unheated WPI control. The WPI-MD6 that was dry heated for 24 h had a one hundred-fold increase in the level of FUR, compared to the respective unheated control, providing evidence for the covalent attachment of maltodextrin to the whey protein molecules during heating. Heat treatment of proteins may result in the  $\beta$ -elimination of seryl or cystyl residues leading to the formation of dehydroalanine, which can then react with the  $\alpha$ -amino group of lysine to produce lysinoalanine (LAL). Protein crosslinking due to the formation of LAL can cause a loss of functional properties and nutritional quality (Pellegrino *et al.*, 1999).

**Table 5.1.** Concentration of furosine, lysinoalanine, pH 4.6 soluble protein and apparent viscosity of unheated solutions and solutions prepared from whey protein isolate (WPI) and WPI-maltodextrin (WPI-MD6) that had been dry heated at an initial pH of 7.0 at 60°C and 79% relative humidity for 24 h. Values are presented as mean  $\pm$  standard deviation of data from three independent trials. Values within a column not sharing a common superscript differ significantly ( $P \leq 0.05$ ).

Sample	Heating Time	Furosine	Lysinoalanine	pH 4.6 soluble protein	Viscosity
	(h)	mg 100 g <sup>-1</sup> protein	mg 100 g <sup>-1</sup> protein	% of total protein	mPa.s
WPI	0	12.7 $\pm$ 2.5 <sup>ab</sup>	4.7 $\pm$ 0.1 <sup>a</sup>	92.8 $\pm$ 1.8 <sup>a</sup>	1.3 $\pm$ 0.0 <sup>a</sup>
	24	17.9 $\pm$ 2.8 <sup>b</sup>	11.4 $\pm$ 1.2 <sup>c</sup>	88.1 $\pm$ 4.3 <sup>a</sup>	1.5 $\pm$ 0.1 <sup>a</sup>
WPI-MD6	0	10.4 $\pm$ 0.3 <sup>a</sup>	4.5 $\pm$ 0.3 <sup>a</sup>	91.8 $\pm$ 1.7 <sup>a</sup>	1.3 $\pm$ 0.0 <sup>a</sup>
	24	1110 $\pm$ 12 <sup>c</sup>	7.5 $\pm$ 0.2 <sup>b</sup>	93.2 $\pm$ 2.6 <sup>a</sup>	1.4 $\pm$ 0.1 <sup>a</sup>

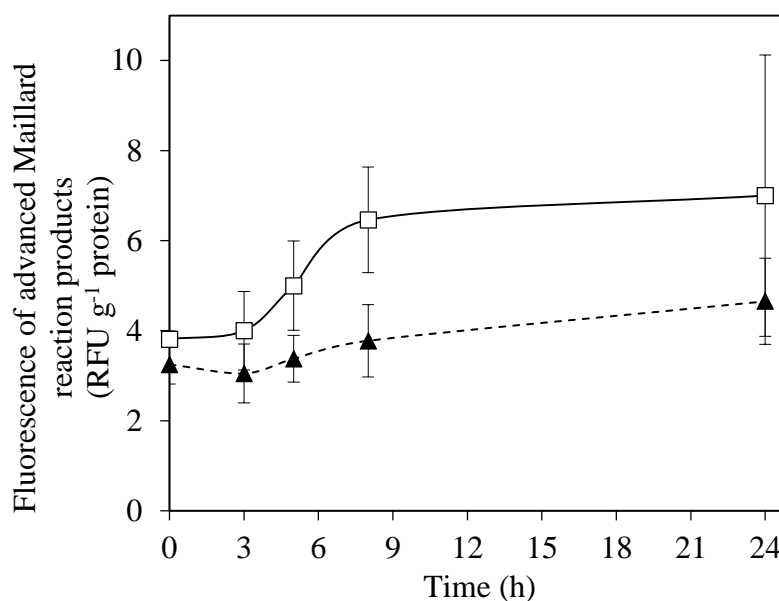
The levels of LAL in the unheated WPI and unheated WPI-MD6 were relatively low (4.5-4.7 mg 100 g<sup>-1</sup> protein; Table 4.1); however, the level of LAL in the dry heated WPI (11.4 mg 100 g<sup>-1</sup> protein) was significantly ( $P \leq 0.05$ ) higher than the level of LAL in the WPI-MD6 dry heated for 24 h (7.5 mg 100 g<sup>-1</sup> protein). In a study by Mulcahy *et al.* (2016a), conjugated solutions of WPI-MD6, produced by heating solutions (5% protein, 5% maltodextrin) at initial pH 8.2, at 90°C for 8 h, had lower levels of LAL than WPI solutions heated alone under the same conditions, due to competition for the  $\epsilon$ -amino group of lysine as a result of their consumption due to covalent attachment of maltodextrin.

#### 5.4.3 Fluorescence of advanced Maillard reaction products

The fluorescence of advanced Maillard reaction products ( $F_{AMRP}$ ) in the unheated WPI was relatively low (3.25 RFU) and did not change significantly ( $P > 0.05$ ) on dry heating for up to 24 h (Fig. 5.2). The formation of advanced Maillard products in the WPI-MD6 sample increased up to 8 h of heating, with only a slight increase in  $F_{AMRP}$  between 8 h (6.5 RFU) and 24 h (7.1 RFU) of dry heating. Leclère and Birlouez-Aragon (2001) reported similar levels of  $F_{AMRP}$  (~10 RFU) in  $\beta$ -lg-lactose solutions heated at 60°C for 70 h.

Cuzzoni *et al.* (1988) reported that for glucose-lysine samples heated (100°C for 30 min) at different water activities ( $a_w$  of 0.98, 0.84 or 0.60), the highest levels of advanced Maillard reaction products were produced at  $a_w$  of 0.84, as the glucose





**Fig. 5.2.** Fluorescence of advanced Maillard products, expressed in relative fluorescence units per gram of protein ( $\text{RFU g}^{-1} \text{ protein}$ ) in the pH 4.6-soluble fraction of solutions prepared from whey protein isolate (WPI;  $\blacktriangle$ ) and WPI-maltodextrin (WPI-MD6;  $\square$ ) dry heated at an initial pH of 7.0 at  $60^\circ\text{C}$  and 79% relative humidity for up to 24 h. Values are presented as mean  $\pm$  standard deviation of data from three independent trials.

and lysine molecules were able to interact without the inhibitory effect of high water content on the initial condensation step (i.e., formation of Schiff base) of the Maillard reaction.

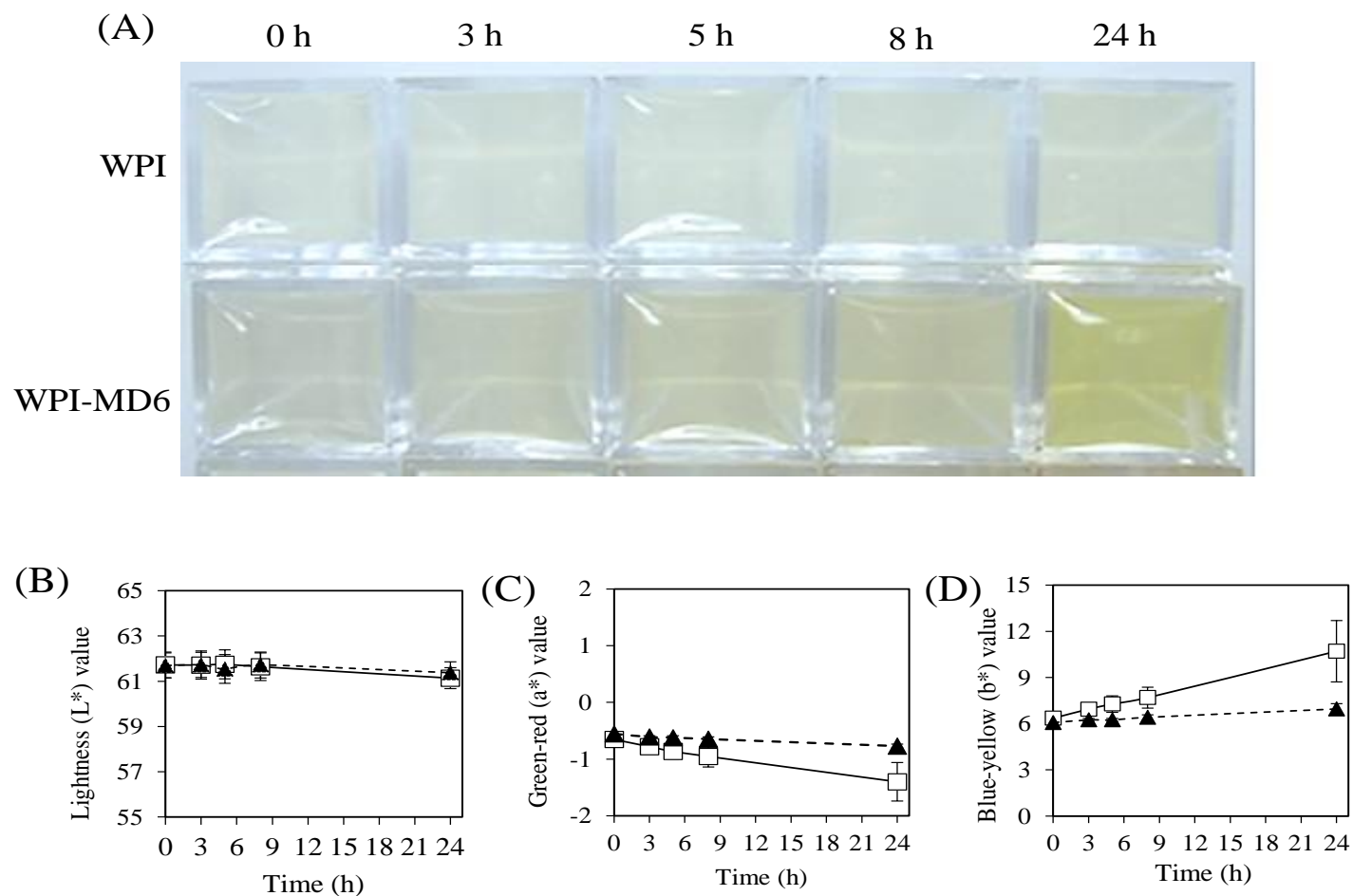
#### 5.4.4 Colour Development

Photographs of colour development in solutions prepared from the WPI and WPI-MD6 throughout the 24 h dry heating time course are shown in Fig. 5.3A. There were no significant ( $P > 0.05$ ) changes in the  $L^*$  values of the solutions prepared from the dry heated WPI and WPI-MD6, or in  $a^*$  and  $b^*$  values of the solution

prepared from the dry heated WPI over the 24 h of heating (Fig. 5.3 B-D). However, there were significant changes in the  $a^*$  and  $b^*$  values of the solution prepared from the dry heated WPI-MD6; there was a significant ( $P \leq 0.05$ ) decrease in the  $a^*$  value and an increase in the  $b^*$  value on heating for 24 h, relative to the unheated control, indicating that yellow colour had developed. This can be attributed to the formation of intermediate-stage Maillard reaction products or nitrogenous polymers and copolymers, known as melanoidins at the advanced stage of the Maillard reaction, which can be yellow or brown in colour (Ames, 1998; Hodge 1953).

#### 5.4.5 Characterisation of protein profile of WPI and WPI-MD6 conjugate by sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The characteristic whey protein bands were evident in unheated WPI and WPI-MD6, including minor whey proteins and monomers of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin (Fig. 5.4 A and B, lanes 2 and 3). On dry heating of the WPI for 24 h (Fig. 5.4 A, lane 4), the intensity of the  $\beta$ -lactoglobulin ( $\beta$ -lg) and  $\alpha$ -lactalbumin ( $\alpha$ -lac) monomer bands decreased slightly in the non-reducing gel, and there was some evidence of formation of protein aggregates ( $>70$  kDa). Similar results were reported by Muhammad *et al.* (2011) for dry heated (at an initial pH of 6.5, at 100°C for 24 h)  $\beta$ -lg, where the intensity of the bands corresponding to monomers of  $\alpha$ -lac and  $\beta$ -lg decreased and large ( $>67$  kDa) aggregates were evident in a non-reducing SDS-PAGE gel, relative to the unheated control. In the reducing gel, the dry heated WPI (Fig. 5.4 B, lane 4) had a similar electrophoretic pattern to the unheated WPI (Fig. 5.4 B, lane 2). In contrast, there was a distinct shift to a broad range of higher molecular weight material for the dry heated conjugated WPI-MD6 (Fig. 5.4 A and



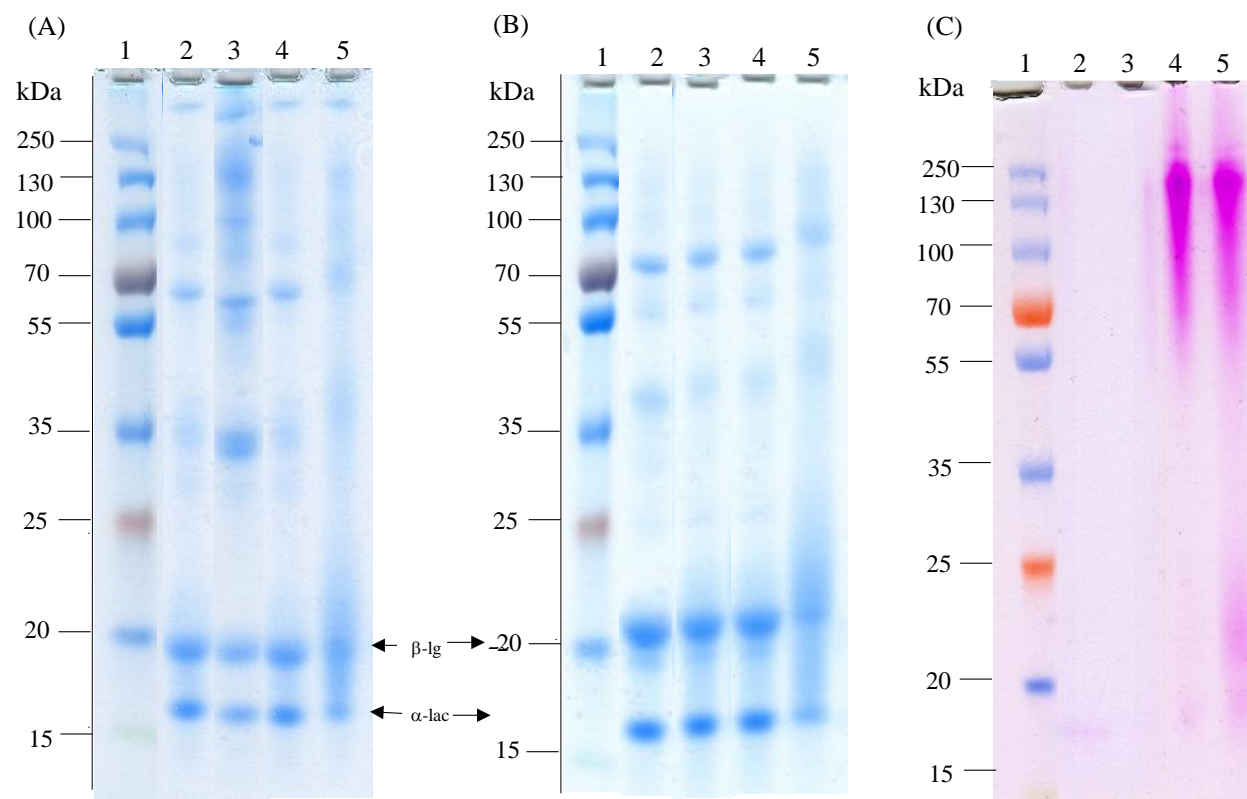
**Fig. 5.3.** Photographic images (A) and colour chromaticity co-ordinates, expressed as  $L^*$  (lightness; B),  $a^*$  (green-red; C) and  $b^*$  (blue-yellow; D) values of solutions prepared from whey protein isolate (WPI;  $\blacktriangle$ ) and WPI-maltodextrin (WPI-MD6;  $\square$ ) dry heated at an initial pH of 7.0 at 60°C and 79% relative humidity for up to 24 h. Values are presented as mean  $\pm$  standard deviation of data from three independent trials.

B, lane 5) compared to the dry heated WPI (Fig. 5.4A and 5B, lane 4) in the reducing gel, indicating the formation of higher molecular weight material mediated by covalent bonds other than disulphide bonds.

No carbohydrate staining was visible in the unheated or dry heated WPI (Fig. 5.4C, lane 2 and 3); however, the maltodextrin in the unheated WPI-MD6 solution (Fig. 5.4C, lane 4) displayed limited migration into the gel. This was possibly due to some starch chains binding with SDS, resulting in the formation of starch-SDS inclusion complexes, which can cause migration of starch chains through SDS-PAGE gels, as shown by Debet and Gidley (2006) and Mulcahy *et al.* (2016a). The maltodextrin component associated with the conjugated protein in the dry heated WPI-MD6 migrated further (than the maltodextrin in the unheated WPI-MD6) through the gel, due to its covalent attachment to the whey proteins. Zhu *et al.* (2008) reported similar results in heated (pH 6.5, 60°C, 24 h) WPI-dextran systems, where the dextran migrated further through the gel compared with the respective unheated control, as it was covalently conjugated to protein.

#### 5.4.6 *Changes in pH 4.6-soluble protein, particle size distribution and viscosity on heating*

The levels of pH 4.6-soluble protein in the solutions prepared from the dry heated WPI and WPI-MD6 were not significantly ( $P > 0.05$ ) different to the levels in the respective unheated controls (Table 4.1). The low levels of protein precipitated at pH 4.6 in the unheated controls was attributed to some denaturation of whey protein during the manufacture of the ingredient.



**Fig. 5.4.** Non-reducing (A) and reducing (B and C) one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoretograms (SDS-PAGE) stained for protein (A and B) with Coomassie Brilliant Blue G-250 and for carbohydrate (C) with periodic acid-Schiff reagent. The lane identification for gels is as follows: molecular weight marker, lane 1; WPI, lanes 2 and 3; WPI-MD6, lanes 4 and 5. Lanes 2 and 4 are unheated WPI and WPI-MD6, respectively; lanes 3 and 5 are WPI and WPI-MD6, respectively, dry heated at an initial pH of 7.0 at 60°C and 79% relative humidity for 24 h.

Abbreviations:  $\beta$ -lg,  $\beta$ -lactoglobulin;  $\alpha$ -lac,  $\alpha$ -lactalbumin.

Similar levels of pH 4.6 soluble protein (~95%) have been reported previously for WPI and this denaturation was attributed to the unit operations involved in the production of the WPI ingredient (Norwood *et al.*, 2016a,b).

The solutions prepared from unheated WPI and WPI-MD6 powders had bimodal particle size distributions with two distinct peaks (Fig. 5.5, Table 5.2); the particles in the first peak (i.e., peak 1 corresponding to smaller size material) had volume-based mean diameters (VMD) of 10.2 and 9.2 nm, respectively, which accounted for 94.8-99.2% of the total peak area. The second peak in the unheated WPI and WPI-MD6 solutions had a VMD of 183 and 173 nm, respectively, which accounted for 0.8-5.2% of total peak area, and most likely represented protein aggregates. Dry heating of WPI for 24 h resulted in a broadening of the particle size distribution profile and an increase in the VMD of the first and second peaks to 13.4 and 294 nm, respectively, indicative of some protein structural/conformational changes.

In a previous study, Gulzar *et al.* (2011) reported that the application of dry heat to  $\beta$ -lg (pH 6.5, 23% RH, heated at 100°C for up to 24 h) resulted in the formation of small protein aggregates (mainly dimers and oligomers). Dry heating of the WPI-MD6 for 24 h resulted in a smaller VMD for peak 1 and 2 (of 12.0 and 189 nm, respectively) compared to the dry heated WPI. In agreement with this finding, Wang, He, Labuza and Ismail (2013) reported that the covalent attachment of dextran to whey proteins limited some protein-protein interactions and protein aggregation on heat treatment compared to the unconjugated whey protein control. The solutions prepared from the unheated WPI and WPI-MD6 had a low initial

apparent viscosity which did not significantly ( $P > 0.05$ ) increase on dry heating (Table 5.1).

#### 5.4.7 Changes in microstructure in solutions on heating

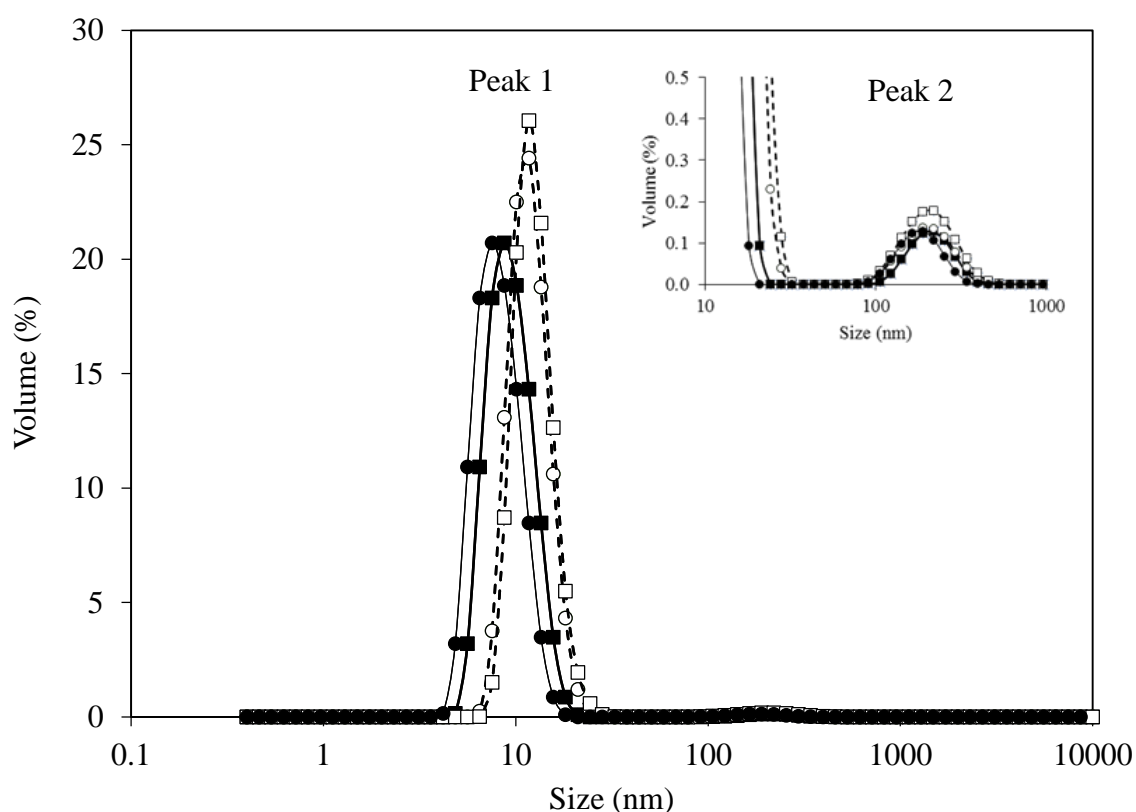
In keeping with the particle size distribution data, transmission electron microscopy (TEM) showed that small roughly spherical particles (~5-10 nm) were present in the unheated WPI and WPI-MD6 solutions (Fig. 5.6A and B). Zhu *et al.* (2008) showed similar TEM micrographs for unheated solutions of WPI, where very small roughly-spherical particles with a diameter of ~3-5 nm were present. Maltodextrin (Fig. 5.6C), stained using uranyl acetate, had the same electron density as the blank grid after specimen preparation and therefore was visualised as a continuous zone of grey on the micrograph.

Solutions prepared from the dry heated WPI contained particles, which varied considerably in size (~5-250 nm; Fig. 5.6 D) and included large, rough-edged spherical structures, indicating that some protein aggregation had taken place. Mudgal *et al.* (2010) showed negatively stained TEM micrographs of heated  $\beta$ -lg solutions (pH 7.0, heated at 85°C for 3 h) and reported similar results where repeating globular structural subunits were visible which connected to form a larger network of aggregates. No large aggregated particles (>~50 nm) were present in the conjugated WPI-MD6 solution (Fig. 5.6E), although the structure of the small spherical particles were lacked definitive edges indicating that maltodextrin, that did not stain, may have associated with the protein.

**Table 5.2.** Particle size distributions for unheated solutions and solutions prepared from powders that were dry heated at an initial pH of 7.0 at 60°C and 79% relative humidity for 24 h; whey protein isolate (WPI), WPI-maltodextrin (WPI-MD6). Values are presented as mean  $\pm$  standard deviation of data from three independent trials.

Sample	Heating Time	Total	Peak 1		Peak 2	
		Average Volume Diameter	Average Volume Diameter	Percent of Total Area	Average Volume Diameter	Percent of Total Area
	(h)	(nm)	(nm)	(%)	(nm)	(%)
WPI	0	12.6 $\pm$ 3.5	10.2 $\pm$ 1.7	94.8 $\pm$ 1.0	183 $\pm$ 9	5.2 $\pm$ 1.0
	24	29.7 $\pm$ 2.8	13.4 $\pm$ 1.9	92.1 $\pm$ 0.4	294 $\pm$ 15	7.9 $\pm$ 0.4
WPI-MD6	0	10.4 $\pm$ 2.3	9.2 $\pm$ 1.7	99.2 $\pm$ 0.1	173 $\pm$ 32	0.8 $\pm$ 0.1
	24	13.6 $\pm$ 1.3	12.0 $\pm$ 0.8	99.0 $\pm$ 0.3	189 $\pm$ 14	1.0 $\pm$ 0.3

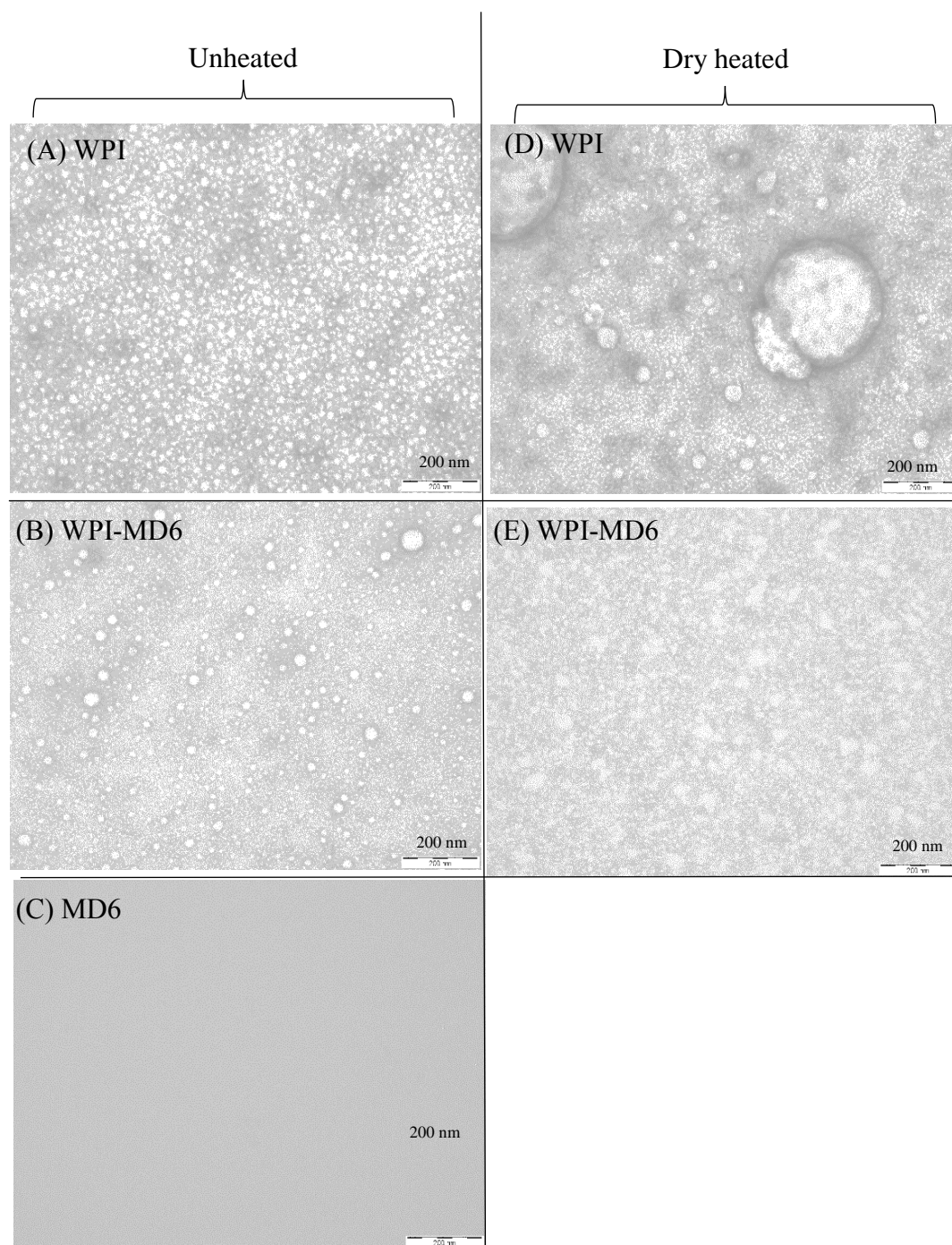




**Fig. 5.5.** Particle size distributions for solutions prepared from unheated WPI and WPI-MD6 (closed symbols) and solutions prepared from powders that were dry heated (open symbols) at an initial pH of 7.0 at 60°C and 79% relative humidity for 24 h; whey protein isolate (WPI; ■, □), WPI-maltodextrin (WPI-MD6; ●, ○). Insert shows data for peak 2 with expanded y axis and contracted x axis.

#### 5.4.8 Protein Solubility

All solutions displayed high protein solubility (>96.2%) at pH 2.0-3.0 and pH 5.5-8.0, with minimum solubility occurring near the isoelectric point of the whey proteins (Fig. 5.7). Dry heating of the WPI for 24 h resulted in an improvement in protein solubility (2.3-5.9% increase) at pH 3.5-4.0, compared to the unheated WPI control, which may be due to modification of protein charge or some conformational



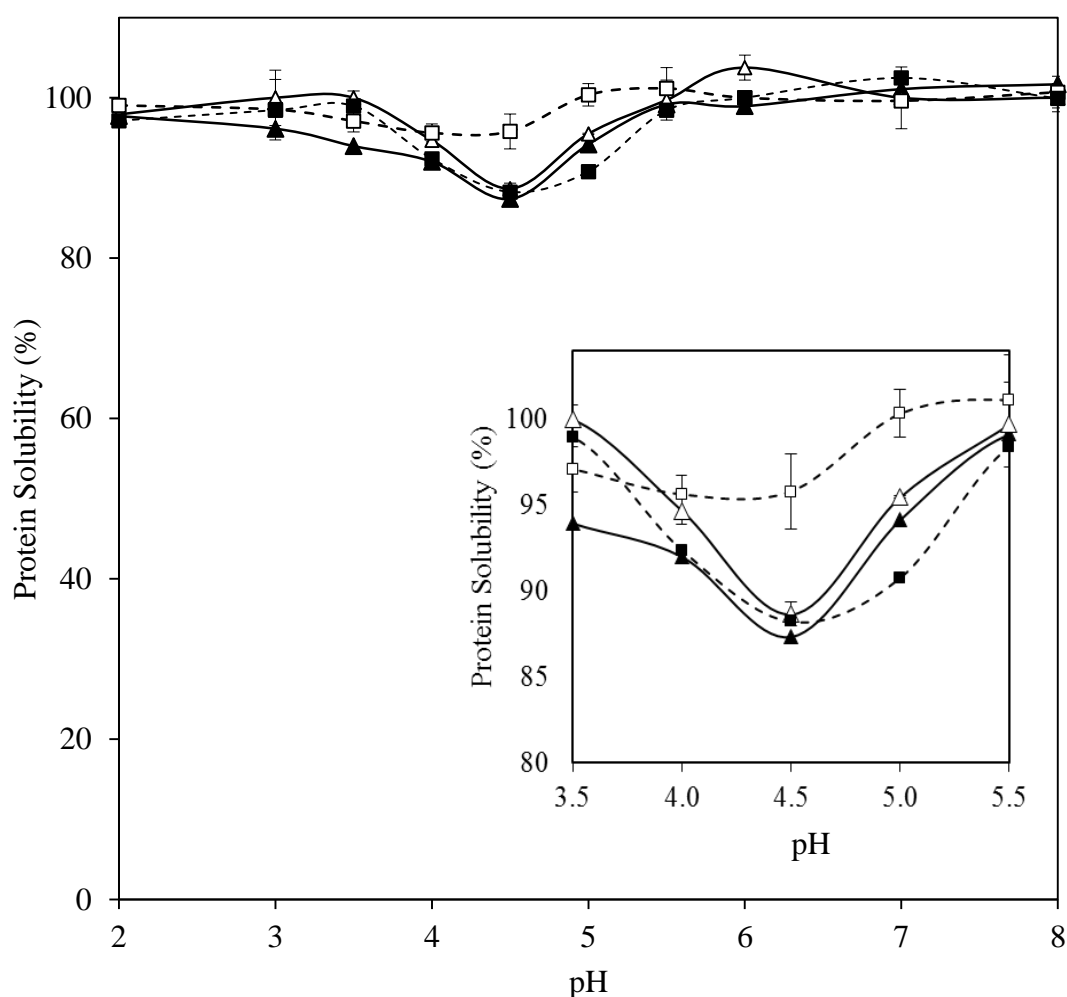
**Fig. 5.6.** Negatively stained transmission electron micrographs of solutions prepared from whey protein isolate (WPI; A, D), maltodextrin (MD6; C) and WPI-MD6 (B, E) mixtures either unheated or dry heated at an initial pH of 7 at 60°C and 79% relative humidity for 24 h. The scale bar = 200 nm.

changes to the proteins on heating, resulting in a reduction in the extent of exposure of hydrophobic regions (Ryan *et al.*, 2012, 2013).

Solutions prepared from the conjugated WPI-MD6 (after 24 h of dry heating) had significantly ( $P \leq 0.05$ ) higher protein solubility (5.0-8.4% increase) at pH 4.5-5.0, compared to unheated WPI, unheated WPI-MD6 and dry heated WPI. Solutions prepared from the conjugated WPI-MD6 also had higher protein solubility at pH 4.0 (95.8% protein solubility) compared to unheated WPI and WPI-MD6 (92.0 and 92.3% protein solubility, respectively). Akhtar and Dickinson (2007) reported improved solubility, particularly at pH 5.0 of WPI after conjugation with maltodextrin by dry heating at 90°C for 2 h and attributed this to reduced protein-protein interactions due to the attachment of the bulky hydrophilic maltodextrin moiety to the protein.

#### 5.4.9 Effect of heating with added NaCl on turbidity development

The solutions (5% protein) prepared from the WPI and WPI-MD6 powders that were not dry heated (Fig 4.8 A1 and 3, respectively) and the solutions prepared from the dry heated WPI and WPI-MD6 powders (Fig 4.8 B1 and 3, respectively) remained transparent when 50 mM NaCl was added and the pH adjusted to 6.8. Once these solutions were heated at pH 6.8, at 85°C, for 10 min, they increased in turbidity (Fig. 5.8 A2, B2, A4, and B4). It is known that the presence of NaCl in protein solutions can decrease the inter-protein charge repulsions, leading to increased protein-protein interactions (Ryan *et al.*, 2012). The solution prepared from the previously unheated WPI powder became very turbid (i.e., black lines of background obscured through the solution, Fig. 5.8 A2) once heated at 85°C for 10 min;



**Fig. 5.7.** Protein solubility as a function of pH for whey protein isolate (WPI) and WPI-maltodextrin (MD6) solutions unheated (closed symbols) or dry heated at an initial pH of 7 at 60 °C and 79% relative humidity for 24 h (open symbols); WPI ( $\blacktriangle$ ,  $\triangle$ ) and WPI-MD6 ( $\blacksquare$ ,  $\square$ ). Values are presented as mean  $\pm$  standard deviation of data from three independent trials. Insert shows data with contracted y axis and expanded x axis.

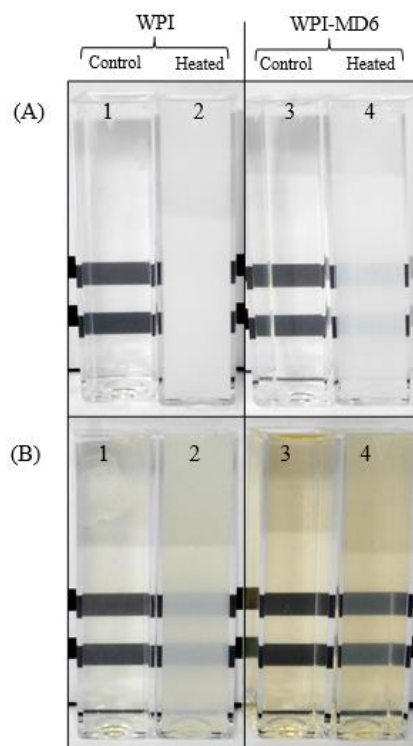
this was accompanied by an 84.1% reduction in the transmission of light through the solution, compared with the unheated control, which was attributed to the formation of large protein aggregates that scattered light. Once heated at 85°C for 10 min, the solution prepared from the previously unheated WPI-MD6 powder also became

turbid and the transmission of light through this solution was reduced by 60.3% (Fig 4.8 A4). The presence of free maltodextrin in this solution may have decreased the amount of water available for hydration of whey protein molecules during heating, reducing the hydrodynamic diameter of protein particles (Table 5.2), therefore reducing the development of turbidity. Chen and O'Mahony (2016) reported that when maltodextrin (5%) was added to an unheated milk protein concentrate solution (8.5% protein), there was a significant ( $P \leq 0.05$ ) decrease in the hydration of the proteins.

The protein solution prepared from WPI powder that was dry heated and had 50 mM NaCl added appeared turbid when it subsequently received a further heat treatment (Fig. 5.8 B2) accompanied by a 36.4% reduction in the transmission of light through the solution, compared to its control (Fig. 5.8 B1). Pre-heating of whey protein has been shown to increase the stability of the protein to subsequent heat treatments by modulating the aggregate shape, surface charge and surface hydrophobicity (Ryan *et al.*, 2013). The solution prepared from the dry heated WPI-MD6 powder that had 50 mM of NaCl added remained transparent (i.e., black lines of background not obscured through the solution, Fig. 5.8 B4) once heated at 85°C for 10 min and there was a very small (11.3%) decrease in the transmission of light through the solution, compared to its control (Fig. 5.8 B3).

Liu and Zhong (2012) reported that solutions prepared from WPI-maltodextrin (dextrose equivalent of 18) dry heated at initial pH 7.0, at 60°C and 80% relative humidity for 24 h (i.e., conjugated) with 100 mM added NaCl, were stable when subsequently heated at 88°C for 2 min as the solution remained transparent. These results indicate that covalent attachment of the bulky hydrophilic MD to the whey protein through Maillard-induced conjugation greatly enhanced

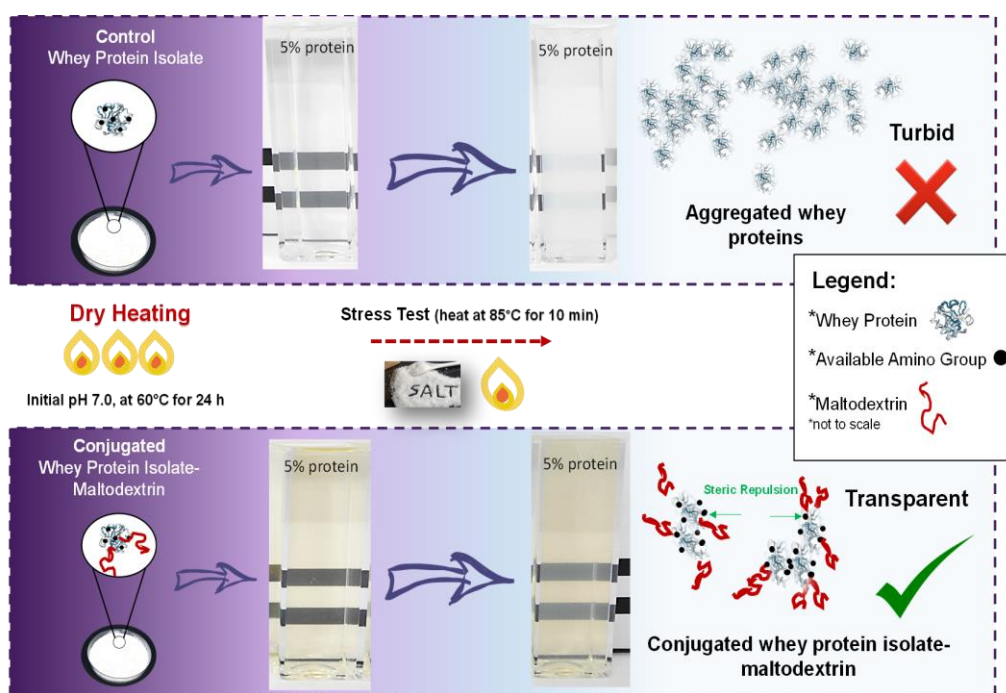
stability of the whey protein solutions when subsequently thermally treated in the presence of NaCl.



*Fig. 5.8. Photographs of whey protein isolate (WPI; 1 and 2) and WPI-maltodextrin (WPI-MD6; 3 and 4) solutions, pH 6.8, containing 5.0% protein and 50 mM added NaCl (Control), and after heating at 85°C for 10 min (Heated). The WPI and WPI-MD6 powders used to prepare solutions shown in (A) received no prior heat treatment (unconjugated), while the WPI and WPI-MD6 powders used to prepare the solutions (B) were previously dry heated at 60°C and 79% relative humidity for 24 h (to induce conjugation).*

This increase in the thermal stability of the conjugated WPI-MD6 is most likely due to enhanced steric hindrance due to the attachment of maltodextrin, thus, minimising protein-protein interactions that help to prevent further heat-induced destabilisation, even in the presence of NaCl (Fig. 5.9). Similar results were discussed by Drapala *et al.* (2016) who reported improved thermal stability (100°C for 15 min) of model infant formula emulsions prepared with a hydrolysed whey protein-maltodextrin conjugate solution, compared with heated model infant formula emulsions

containing lipid-based emulsifiers (i.e.,  $\leq 9 \text{ g L}^{-1}$ , lecithin or CITREM); the authors attributed this to increased steric stabilisation of the protein molecules and reduced interactions between proteins/peptides adsorbed at the interfaces of oil droplets.



**Fig. 5.9.** Schematic diagram illustrating conjugated whey protein-maltodextrin solutions stabilised by steric repulsion mechanisms.

## 5.5 Conclusion

The dry heating conditions used in this study (initial pH 7.0, heated at 60°C, 79% RH for 24 h) facilitated conjugation of maltodextrin to whey protein molecules, while restricting whey protein denaturation and limiting the progression of the Maillard reaction to advanced stages. Conjugation of WPI-MD6 for 24 h increased the protein solubility at pH 4.5 by 8.5%, compared to the unheated WPI sample. Conjugation of WPI with MD6 enhanced the stability (i.e., maintained solution clarity) to thermal treatment of whey protein solutions (5.0%, w/v, protein) at 85°C for 10 min with 50 mM added NaCl, compared to unheated and heated WPI controls, due to increased steric repulsion. This study demonstrated that conjugation of whey protein with maltodextrin has the potential to produce ingredients that can be used in the production of nutritional beverages with enhanced thermal stability. Further work is required to optimise the functionality of these ingredients in formulated food systems.

## 5.6 Acknowledgements

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## Chapter 6

### *Improvement of the functional properties of whey protein hydrolysate by conjugation with maltodextrin*

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## 6.1 Abstract

The impact of conjugation with maltodextrin on selected functional properties (i.e., solubility and thermal stability) of intact whey protein isolate (WPI) and whey protein hydrolysate (WPH) was determined. Conjugation of WPI and WPH (degree of hydrolysis 9.3%) with maltodextrin (MD) was achieved by heating solutions of 5% WPI or WPH with 5% MD, initial pH 8.2, at 90°C for up to 24 h. The WPH had 55.4% higher levels of available amino groups compared with the WPI, which contributed to more rapid and extensive conjugation of WPH-MD, compared with WPI-MD. The WPI-MD and WPH-MD solutions heated for 8 h had significantly higher ( $P \leq 0.05$ ) protein solubility than the respective WPI and WPH heated control solutions, in the pH range 4.0-5.0. Conjugation of WPI and WPH with MD enhanced the stability to heat-induced changes, such as turbidity development, gelation or precipitation, in the presence of 40 mM added NaCl. Conjugation with MD is an effective means of enhancing the physicochemical functionality of whey proteins, with hydrolysis of the whey protein substrate resulting in faster and more extensive conjugation of the proteins/peptides.



## 6.2 Introduction

The Maillard reaction was first described by the French biochemist, Louis-Camille Maillard, in 1912 and is initiated when the  $\epsilon$ -amino groups of lysyl residues and the  $\alpha$ -amino groups of terminal amino acids in proteins condense with the carbonyl groups of reducing carbohydrates (Martins *et al.*, 2000). The reaction pathways of the Maillard reaction at the intermediate and advanced stages are complex and involve polymerisations, cyclisations and enolisations to form a diverse mixture of compounds (Hodge, 1953). The Maillard reaction pathways and the compounds formed are dependent on many factors including temperature, time, pH, water activity and the intrinsic properties of the proteins and carbohydrate (Liu *et al.*, 2012). Several studies have reported that conjugation of carbohydrate to protein, at the early stages of the Maillard reaction, can improve the functionality of the protein (Davidov-Pardo *et al.*, 2015; Liu & Zhong, 2015; Martinez-Alvarenga *et al.*, 2014).

Whey protein ingredients are commonly used in foods to exploit their nutritional and functional properties. The incorporation of whey protein hydrolysates into food formulations is a growing area of interest as they can provide specialised nutritional support for those with particular physiological needs (Clemente, 2000). In some nutritional products, whey protein hydrolysates are primarily used to reduce antigenicity associated with the intact protein and to increase tolerance and digestibility of the proteins in milk protein-sensitive consumers (Nnanna & Wu, 2007). Protein hydrolysates are generally characterised by their degree of hydrolysis (DH), which expresses the number of peptide bonds cleaved as a percentage of the total number of peptide bonds available (Foegeding *et al.*, 2002). Controlled

hydrolysis of whey proteins alters their physicochemical functionalities, including the solubility, heat stability, viscosity, emulsifying and foaming properties (Singh & Dalgleish, 1998; Wijayanti *et al.*, 2014).

During heating, particularly in high ionic strength environments, intact whey proteins are prone to denaturation and aggregation, which can result in physical destabilisations, manifested as increased turbidity, gelation or phase separation of formulated products containing intact whey protein. Ju *et al.* (1995) reported that limited hydrolysis of whey protein isolate (DH 2-7%), using trypsin, prevented heat-induced gelation of a protein solution (12%, w/v, protein) on heating at 80°C for 30 min at pH 3 and 7; the low level of enzymatic hydrolysis liberated peptides with a lower molecular mass and less secondary structure than the intact proteins, which limited heat-induced aggregation and gelation. However, whey protein hydrolysates can be susceptible to destabilisation when heated due to the exposure of buried hydrophobic residues and/or release of specific peptides that promote peptide-peptide and peptide-protein aggregation (Adjonu *et al.*, 2013; Creusot & Gruppen, 2007a).

There is an increasing global demand for hydrolysed whey protein ingredients; however, hydrolysis of whey proteins may result in impairment of their functional properties (Dairymark, 2012; Smithers, 2008). O'Regan and Mulvihill (2013) reported that conjugation of sodium caseinate hydrolysates (DH 6, 13 or 48%) with maltodextrin conferred improved emulsion stability under accelerated shelf life testing conditions (7 d at 45°C) and enhanced protein solubility (by ~10-50%) at pH 4.0-5.5, compared with the unconjugated sodium caseinate hydrolysates. It appears that there are no reports published on the functional properties of conjugated whey

protein hydrolysates, or the influence of hydrolysis of whey protein on the extent of Maillard-induced conjugation. The present study focused on the influence of conjugation on selected functionalities (i.e., solubility and solution clarity/stability to heating with added salt) of intact and hydrolysed whey proteins. A reaction time course of up to 24 h was chosen for the study as it is beneficial to limit the progression of the Maillard reaction as conjugation occurs at the early stages of the Maillard reaction and advanced Maillard reaction products may cause off-flavours, loss of nutritional value, protein crosslinking and generation of potentially toxic compounds (Martins *et al.*, 2000). Furthermore, the progression of conjugation of whey protein with maltodextrin was monitored to assess if conjugation was impacted by the physical state of the whey protein (i.e., intact or hydrolysed).

## 6.3 Materials and Methods

### 6.3.1 Materials

Whey protein isolate (WPI; produced by ultrafiltration and microfiltration) and whey protein hydrolysate (WPH; produced from the WPI) were obtained from Carbery Food Ingredients (Ballineen, Co. Cork, Ireland). Protein content of the samples was determined using the Kjeldahl method as outlined in AOAC (1995), using nitrogen to protein conversion factors of 6.38 and 6.46 for WPI and WPH, respectively. Anand *et al.* (2002) reported that different nitrogen to protein conversion factors should be used for intact and hydrolysed whey proteins in order to account for the structural differences between proteins and peptides due to the hydrolysis process (e.g., addition of water molecules to the cleaved peptide molecules during hydrolysis). Ash content was determined by heating samples at 500°C until a white ash was obtained (AOAC, 1995). Lactose was determined as described by Indyk *et al.* (1996) with minor modifications as detailed by Mulcahy *et al.* (2016a). Maltodextrin with a dextrose equivalent value of 17 (MD17) containing 0.1% ash and 4.0% moisture was obtained from Corcoran Chemicals Ltd. (Dublin, Ireland). All other reagents and chemicals were procured from Sigma-Aldrich (Tallaght, Ireland), unless otherwise stated.

### 6.3.2 Size exclusion chromatography

Size exclusion chromatography (SEC) was carried out as described by O'Loughlin *et al.* (2013) with the column eluate was monitored at 214 nm to determine the molecular weight distribution profile of the proteins and peptides in the WPI and WPH ingredients.

### 6.3.3 Preparation of protein-carbohydrate conjugate solutions

Blends of either WPI or WPH (5%, w/v, protein) and MD17 (5%, w/v, carbohydrate), were added to ultrapure water and allowed to solubilise for 2 h, at 22°C, with low speed magnetic stirring. The solutions (WPI/WPH-MD17) were adjusted to pH 8.2 with 0.5 N KOH and allowed to hydrate for 18 h at 4°C, before being readjusted to pH 8.2 with 0.5 N KOH at 22°C. The conjugated protein solutions were prepared according to the method of Mulcahy *et al.* (2016a); briefly, aliquots (250 mL) of each solution were placed in 500 mL screw-capped, glass, conical flasks and heated at 90°C for up to 24 h in a pre-equilibrated, shaking water bath. Samples were removed after heating for 3, 5, 8 and 24 h and cooled immediately in iced water and stored at 4°C for further analysis. Control solutions of either WPI or WPH (5%, w/v, protein) were also prepared, heated and sampled as described above.

### 6.3.4 Determination of degree of hydrolysis and available amino groups

The degree of hydrolysis (the number of peptide bonds cleaved enzymatically and expressed as a percentage of the original number of peptide bonds) of the WPH ingredient and the available amino groups (AAG) of all unheated and heated WPI/WPH and WPI/WPH-MD17 solutions were quantified by the o-phthalaldehyde (OPA) method as described by Nielsen *et al.* (2001) with minor modifications as described by Mulcahy *et al.* (2016a).

### 6.3.5 Determination of lysinoalanine

The level of lysinoalanine (LAL) in unheated and heated WPI/WPH and WPI/WPH-MD17 solutions was quantified by reversed phase ultra-performance liquid chromatography (Acquity H Class, Waters Corporation, Milford, MA, USA) as described in Chapter 4.

### 6.3.6 Fluorescence of advanced Maillard reaction products and soluble tryptophan

The fluorescence of advanced Maillard reaction products ( $F_{AMRP}$ ) was measured as described by Mulcahy *et al.* (2016a). The protein content of the pH 4.6 soluble fraction of each sample was measured using a BCA assay kit (Fisher Scientific, Ballycoolin, Dublin, Ireland). The fluorescence of advanced Maillard reaction products ( $F_{AMRP}$ ) was expressed as relative fluorescence units per gram of protein ( $\text{RFU g}^{-1}$  protein).

### 6.3.7 Measurement of colour

The colour of each solution was measured using a Minolta Chroma Meter CR-colorimeter 400 (Minolta Ltd., Milton Keynes, UK) using CIE colour chromaticity coordinates and expressed as  $L^*$  (light-dark) values. Each solution was loaded into a glass cell (CM-A98, optical path length: 10 mm) which was held in position by means of a transmittance specimen holder (CM-A96) and positioned with a calibration plate behind the glass cell, before  $L^*$  value was measured.

### 6.3.8 Protein Solubility

Protein solubility of WPI/WPH and WPI/WPH-MD17 solutions heated at an initial pH of 8.2 at 90°C for 8 h, and their respective unheated controls, was determined in the pH range 2-8 using the method described by Mulcahy *et al.* (2016a).

### 6.3.9 Effect of heating with added NaCl on physicochemical properties and microstructure

The development of turbidity in solutions was determined based on the method of Liu and Zhong (2015) and Mulcahy *et al.* (2016a). Solutions of unheated WPI/WPH and WPI/WPH-MD17 (2.5%, w/v, protein, pH 6.8, with 40 mM added NaCl) were prepared as controls and each solution (2.5 mL) was subsequently heated at 85°C, in 7 mL polycarbonate tubes (length, 47 mm; external diameter, 20 mm; wall thickness, 2 mm), for 10 min in a water bath before the tubes were cooled by immersion in iced water for 5 min. The solutions of WPI/WPH and WPI/WPH-MD17, previously heated at initial pH 8.2, for 8 h at 90°C (i.e., conditions required to achieve conjugation) were adjusted to pH 6.8, diluted to 2.5%, w/v, protein and 40 mM NaCl was added before being heated at 85°C for 10 min (i.e., treated in the same manner as that described above). The turbidity of the solutions was compared by measuring the transmission of light at 400 nm through the sample in a spectrophotometer (Varian Inc., Palo Alto, CA, USA) and by assessing and recording the changes in visual appearance using photography. Turbidity results are reported as the percentage difference in transmission of light between the respective control solutions and those heated at 85°C for 10 min.

Microstructural analysis of the solutions containing 40 mM added NaCl that were heated (at pH 6.8, at 85°C, for 10 min) and their respective controls, as described previously, was performed using an Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan). Samples were vortexed (5 s) before protein was fluorescently labelled with Fast Green FCF dye (0.1%, w/v) as described by Drapala *et al.* (2015). Visualisation of protein in the solutions (10 µL) was carried out in glass bottom micro-well dishes using a He-Ne laser operating at an excitation wavelength of 633 nm. The observations were performed using 100× oil immersion objectives. At least three specimens of each sample were observed to obtain representative micrographs of samples.

#### 6.3.10 Statistical analysis

The preparation of the WPI/WPH and WPI/WPH-MD17 solutions, and their subsequent analysis, was completed in triplicate across three independent trials. Analysis of variance (ANOVA) was carried out followed by Tukey's mean comparison test to establish the significance of differences among the mean values using the Minitab 16 (Minitab Ltd, Coventry, UK, 2007) statistical analysis package and the level of significance was determined at  $P \leq 0.05$ .



## 6.4 Results and discussion

### 6.4.1 Compositional characterisation of WPI and WPH

Compositional analysis of the WPI and WPH showed that both ingredients had generally similar protein and ash contents but that the lactose content of the WPH was higher than that of the WPI (Table 5.1) and the degree of hydrolysis (9.3%) of the WPH was classified as low (Panyam & Kilara, 1996). Size exclusion chromatography profiles of the WPI showed that 28.0% of total protein had a molecular weight >20 kDa, which was attributed to the presence of minor whey proteins such as bovine serum albumin (BSA; 66 kDa), lactoferrin (78-86 kDa), immunoglobulins (55-59 kDa) or whey protein oligomers/aggregates (Table 5.1). The majority (50.5%) of total protein in the WPI had a molecular weight in the range 10-20 kDa; a range that represents the major native whey protein fractions  $\beta$ -lactoglobulin ( $\beta$ -lg; 18.4 kDa) and  $\alpha$ -lactalbumin ( $\alpha$ -lac, 14.2 kDa). The remaining material (21.5% of total protein) had a molecular weight between 0-10 kDa, which is likely due to the presence of glycomacropeptide (~7.5 kDa; Sharma *et al.*, 2013), small amounts of proteose peptones or other low molecular weight protein components (Saint-Sauveur *et al.*, 2008).

The WPH had 12.0% of total protein with molecular weight >20 kDa which could be attributed to the low abundance of high molecular weight whey proteins (as described above) or protein/peptide oligomers/aggregates formed during the production of the protein hydrolysate (Nielsen, 1997; O'Loughlin *et al.*, 2013). The WPH had 24.2% of total protein in the molecular weight range 10–20 kDa, corresponding mainly to residual intact  $\beta$ -lg and  $\alpha$ -lac. The remaining material (61.9%) had molecular weight in the range 0-10 kDa which may be associated with

peptides. A small amount (1.8%) of insoluble material was present in the WPH, which is likely due to the formation of high molecular weight aggregated whey protein material during heat treatment applied to the protein solution prior to hydrolysis or during the thermal inactivation of proteolytic enzymes in the production of WPH (O'Loughlin *et al.*, 2013).

**Table 6.1.** Composition and degree of hydrolysis of the whey protein isolate (WPI) and whey protein hydrolysate (WPH). Values are presented as mean  $\pm$  standard deviation of data from three independent trials.

Composition	WPI	WPH
	(% w/w)	
Protein	87.2 $\pm$ 0.9	83.7 $\pm$ 0.5
Lactose	0.4 $\pm$ 0.02	2.8 $\pm$ 0.1
Ash	2.8 $\pm$ 0.1	2.9 $\pm$ 0.1 <sup>a</sup>
Degree of hydrolysis <sup>b</sup>	-	9.3 $\pm$ 0.7
<b>Molecular weight profile</b>	<b>% of total protein</b>	
Insoluble	0.0 $\pm$ 0.0	1.8 $\pm$ 0.6
>20 kDa	28.0 $\pm$ 3.4	12.0 $\pm$ 1.6
10-20 kDa	50.5 $\pm$ 3.7	24.2 $\pm$ 8.8
5-10 kDa	4.9 $\pm$ 0.2	9.5 $\pm$ 1.9
2-5 kDa	15.6 $\pm$ 0.3	11.9 $\pm$ 1.6
0-2 kDa	0.9 $\pm$ 0.1	40.5 $\pm$ 2.0

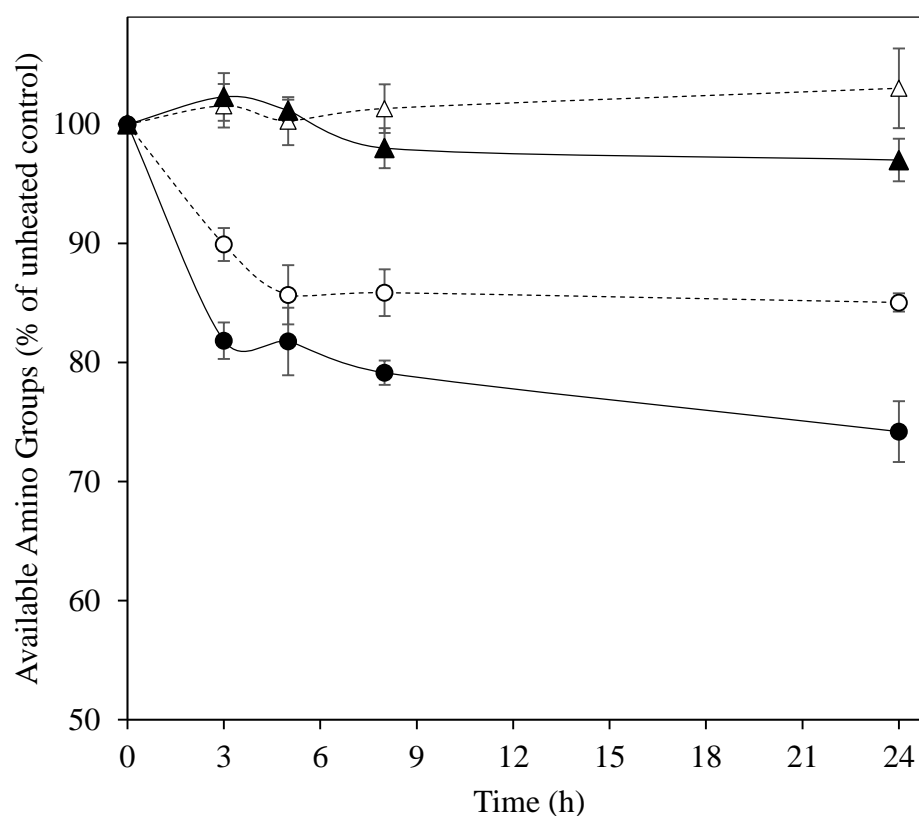
<sup>a</sup>calculated by difference

<sup>b</sup>number of peptide bonds cleaved expressed as a percentage of the original number of peptide bonds

#### 6.4.2 Change in available amino groups on heating

Only the  $\alpha$ -amino group of an N-terminal amino acid or the  $\epsilon$ -amino group of lysyl residues can partake in the Maillard reaction, which amounts to 16 and 13 potential available conjugation sites per  $\beta$ -lg and  $\alpha$ -lac molecule, respectively (Chevalier *et al.*, 2001). During enzymatic hydrolysis, proteins are broken down into peptides and free amino acids (as a result of cleavage of the peptide bonds) which can generate new  $\alpha$ -amino groups and expose previously-buried lysyl residues. The total number of amino groups available to react with the carbonyl groups of reducing sugars was 55.4% higher in the WPH solution than in the WPI solution, giving the WPH a greater propensity to undergo Maillard-induced changes during heating.

The physical state of the protein affected the rate and extent of the reduction of available amino groups (AAG) during heating (Fig. 6.1). The level of AAG in the WPI control solution did not decrease on heating at 90°C for 24 h but there was a 15.0% reduction in AAG for the WPH control solution under the same heating conditions. This decrease in AAG for the WPH solution may have been due to heat-induced structural changes in the protein due to protein/peptide aggregation which could also cause blockage of the AAG within the WPI/WPH solutions (Creusot & Gruppen, 2007a) and differences in reactivities of the amino groups. The WPI/WPH solutions containing MD17 had the greatest reduction in AAG compared to the respective WPI/WPH controls (without MD17). In the WPI-MD17 solution, 24 h of heating was required for the AAG to reduce by 3.0% compared to a 26.0% reduction in AAG for hydrolysed WPH-MD17 solutions after the same treatment. The greatest reduction in AAG for the WPH-MD17 solution



**Fig. 6.1.** Concentration of available amino groups in whey protein isolate (WPI), whey protein hydrolysate (WPH) and WPI/WPH-maltodextrin (MD17) solutions heated at an initial pH of 8.2 at 90°C for up to 24 h, expressed as a percentage of the respective unheated control; WPI (Δ), WPH (○), WPI-MD17 (▲) and WPH-MD17 (●).

occurred during the first 8 h (20.9% reduction in AAG) of heating, with a considerably smaller, further decrease (5.1%) in AAG occurring between 8 and 24 h of heating. At 8 h of heating there was a 2.0% reduction in AAG on conjugation of WPI-MD17 compared to a 21.1% reduction on conjugation of WPH-MD17.

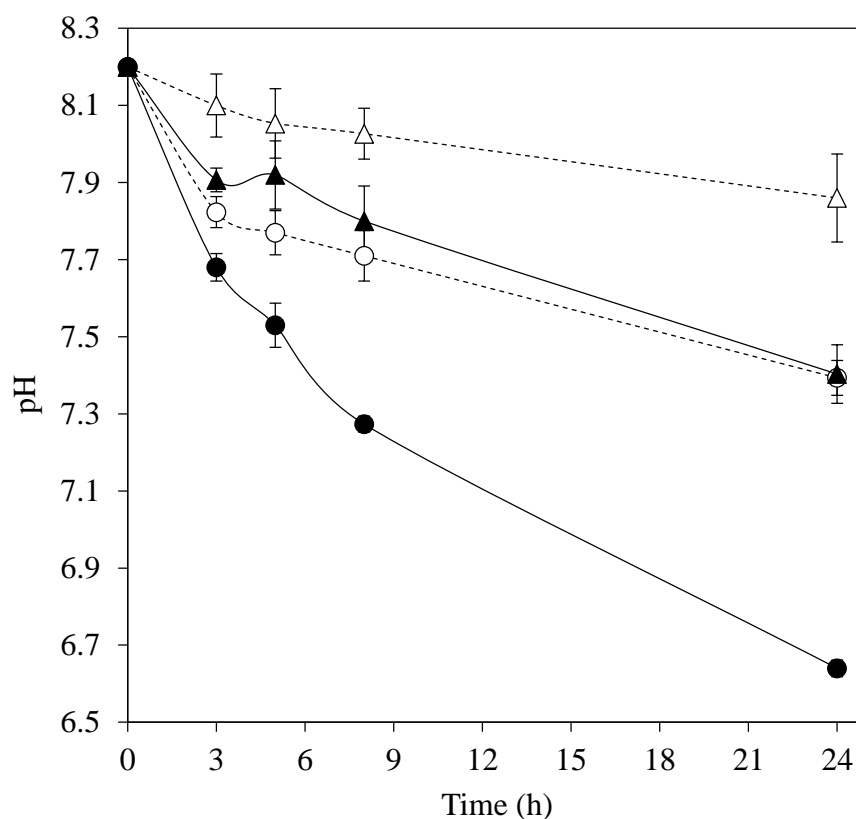
#### 6.4.3 Development of lysinoalanine on heating

The presence of lysinoalanine (LAL) in protein solutions has been reported to be responsible for crosslinking of protein leading to impairment of functional properties and loss of nutritional value (Pellegrino *et al.*, 1999). The WPI solution

heated for 8 h at 90°C had a high level of LAL (179 mg 100 g<sup>-1</sup> protein) compared to the unheated WPI solution (14.1 mg 100 g<sup>-1</sup> protein). The level of LAL in the heated WPI-MD17 solution (58.8 mg 100 g<sup>-1</sup> protein) at 8 h was significantly ( $P \leq 0.05$ ) lower than in the heated WPI solution. However, the level of LAL in the heated WPH solution (100 mg 100 g<sup>-1</sup> protein) at 8 h was not significantly ( $P > 0.05$ ) different from that in the heated WPH-MD17 solution (98.2 mg 100 g<sup>-1</sup> protein) at 8 h. Heat treatment of proteins, especially at alkaline pH, can lead to the formation of LAL by  $\beta$ -elimination of seryl or cystyl residues to form the intermediate dehydroalanine, which can then react with the  $\epsilon$ -amino group of lysine (Calabrese *et al.*, 2009). As the  $\epsilon$ -amino group of lysine can also be consumed by the covalent attachment of maltodextrin to protein during the Maillard reaction (Fig. 6.1), competition for the  $\epsilon$ -amino reaction sites is likely to result in lower levels of LAL in heated WPI/WPH-MD17 solutions compared to the heated WPI solution.

#### 6.4.4 Change in pH on heating

Preliminary trials showed that heating of the WPI/WPH-MD17 solutions at an initial pH of 6.8, at 90°C resulted in gelation or physical destabilisation and separation of the samples; therefore an initial pH of 8.2 was selected to minimize these intermolecular protein-protein/peptide interactions. The pH of the WPI control solution decreased slightly but not significantly ( $P > 0.05$ ) during heating (90°C for up to 24 h) but the pH of the WPI-MD17 solution decreased significantly ( $P \leq 0.05$ ) from pH 8.2 to 7.4 on heating for 24 h (Fig. 6.2). The decrease in pH can be attributed to the production of organic acids (i.e., formic or acetic acid) during the intermediate



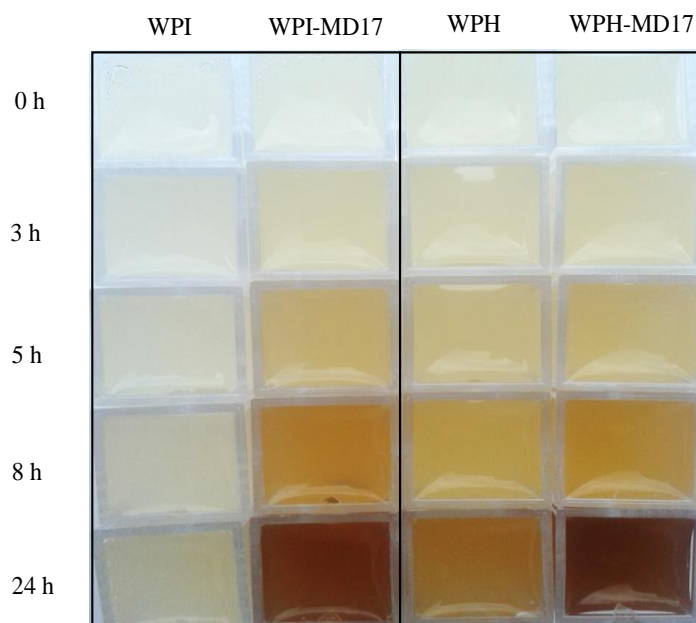
**Fig. 6.2.** pH of whey protein isolate (WPI), whey protein hydrolysate (WPH) and WPI/WPH-maltodextrin (MD17) solutions heated at an initial pH of 8.2 at 90°C for up to 24 h; WPI (Δ), WPH (○), WPI-MD17 (▲) and WPH-MD17 (●).

stages of the Maillard reaction and the consumption of charged amino acids, such as the basic amino acid lysine (Martins *et al.*, 2000). The WPH control solution decreased from pH 8.2 to 7.4 after 24 h of heating which is likely due to the reaction of the proteins/peptides with innate lactose present in the WPH during heating (Berg & Van Boekel, 1994). The pH of the WPH-MD17 solution decreased from 8.2 to 6.6 after 24 h of heating, indicating that the Maillard reaction progressed at a greater rate and to a greater extent than in all other solutions. The decrease in pH of the WPI/WPH-MD17 solutions was most rapid during the first 8 h of heating.

#### 6.4.5 Colour development

Colour development during the Maillard reaction occurs during the advanced stages and is generally attributed to the formation of nitrogenous polymers and co-polymers (Friedman, 1996). Photographs of colour development in all solutions throughout the 24 h heating time course are shown in Fig. 6.3. The  $L^*$  value of the WPI solution decreased from 56.8 to 48.6 units after 24 h of heating at 90°C, most likely due to reaction of the whey proteins with innate lactose (0.4%) present in the WPI ingredient on heating, resulting in low levels of browning (associated with the Maillard reaction). The  $L^*$  value of the WPI-MD17 solution decreased from 56.8 to 34.9 units as brown colour developed after 24 h of heating at 90°C; however, there was only a low corresponding reduction of AAG (3.0%) in the heated WPI-MD17 solution, indicating low levels of conjugation were achieved even as the production of colour compounds associated with the advanced stages of the Maillard reaction was evident.

The  $L^*$  value of the WPH solution decreased from 49.0 to 41.4 units while that of the WPH-MD17 solution decreased from 49.6 to 30.0 units after 24 h of heating with a concomitant development of brown colour. The production of coloured compounds in the WPH/WPI-MD17 solutions at 8 h of heating was limited compared to the solutions at 24 h of heating; at heating times >8 h, there were significant decreases ( $P \leq 0.05$ ) in the  $L^*$  value for all solutions containing maltodextrin, with an accompanying increase in brown colour being evident.



**Fig. 6.3.** Photographic image of whey protein isolate (WPI), whey protein hydrolysate (WPH) and WPI/WPH-maltodextrin (MD17) solutions heated at an initial pH of 8.2 at 90°C for up to 24 h

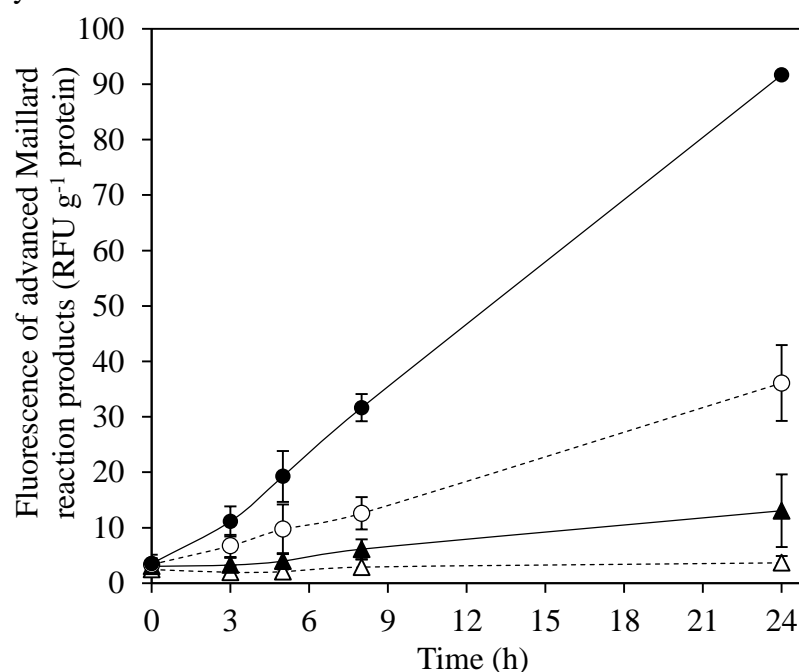
#### 6.4.6 Fluorescence of advanced Maillard reaction products

In order to maximise the conjugation of proteins/peptides with maltodextrin and minimise the progression of the Maillard reaction into the later stages, the fluorescence of advanced Maillard reaction products ( $F_{AMRP}$ ) was monitored throughout the 24 h heating time course (Fig. 6.4). All the unheated solutions of WPI/WPH and WPI/WPH-MD17 had low levels of  $F_{AMRP}$  (2.4-3.5 relative fluorescence units; RFU) and the WPI solution did not significantly ( $P > 0.05$ ) increase in  $F_{AMRP}$  (3.7 RFU) after 24 h of heating at 90°C.

There was relatively limited development of  $F_{AMRP}$  in the WPI-MD17 solution (13.0 RFU) after heating for 24 h. The  $F_{AMRP}$  increased linearly ( $R^2 > 0.98$ ) as a function of heating time for the WPH and WPI/WPH-MD17 solutions. The level



of  $F_{AMRP}$  was significantly ( $P \leq 0.05$ ) higher in both the WPH (36.1 RFU) and the WPH-MD17 (91.7 RFU) solutions when compared to the WPI (3.6 RFU) and WPI-MD17 (13.0 RFU) solutions, respectively, after 24 h of heating. Similar results were reported by Da Silva Pinto *et al.* (2011) who showed that heating solutions containing mixtures of  $\beta$ -Lg and glucose (at an initial pH of 7, at 90°C for 24 h) resulted in a threefold increase in the quantity of  $F_{AMRP}$  in the solutions. The WPI/WPH-MD17 solutions after 8 h of heating, which were found to have significantly ( $P \leq 0.05$ ) lower levels of  $F_{AMRP}$  (6.1 and 31.7 RFU) and colour development, than those heated for 24 h, were thus chosen for assessment of functionality.



**Fig.6.4.** Fluorescence of advanced Maillard reaction products, expressed in relative fluorescence units (RFU) per gram of protein, in the pH 4.6-soluble fraction of whey protein isolate (WPI), whey protein hydrolysate (WPH) and WPI/WPH-maltodextrin (MD17) solutions heated at an initial pH of 8.2 at 90°C for up to 24 h; WPI ( $\Delta$ ), WPH ( $\circ$ ), WPI-MD17 ( $\blacktriangle$ ) and WPH-MD17 ( $\bullet$ ).

#### 6.4.7 Protein Solubility

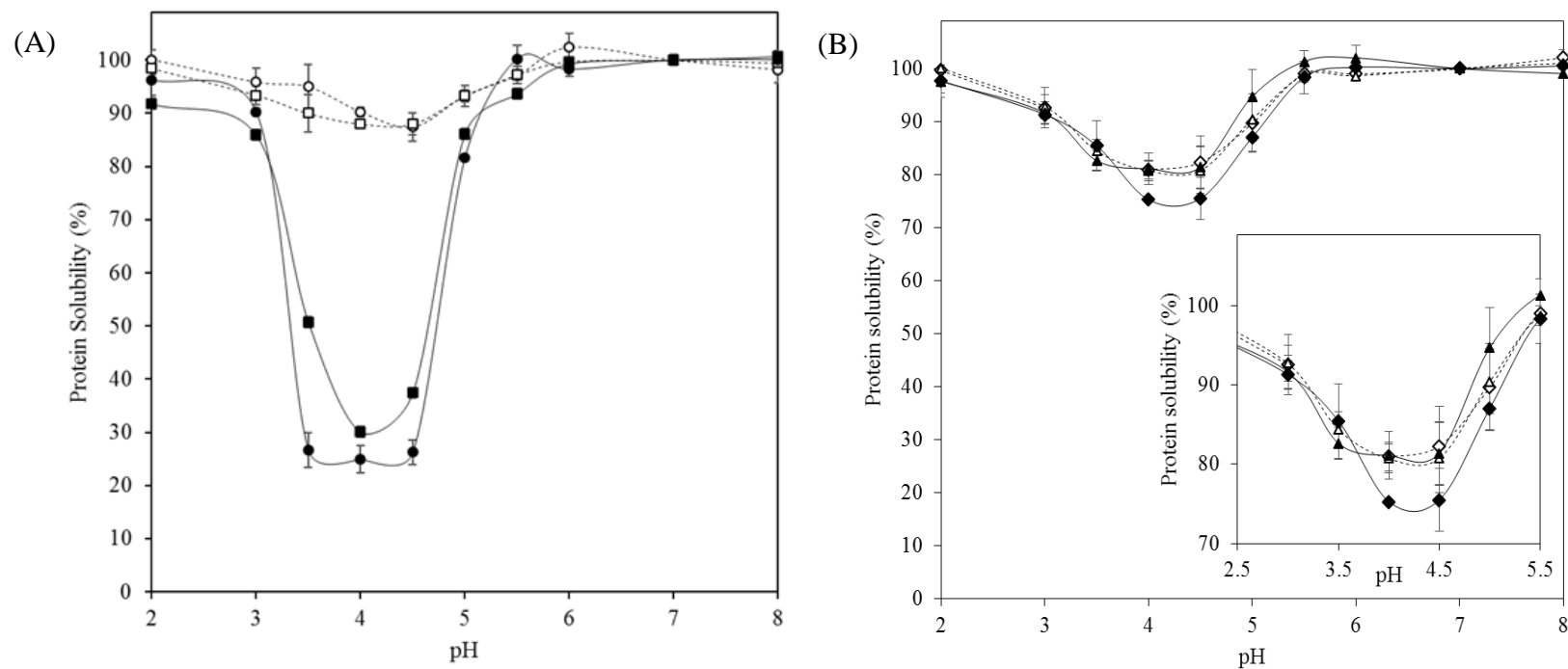
Unheated WPI and WPI-MD17 solutions had high protein solubility ( $\geq 88.0\%$ ) at pH 2.0-8.0 with the lowest protein solubility for the unheated WPI and WPI-MD17 solutions occurring near the isoelectric point of the whey proteins (Fig 5.5A). The protein solubility of the WPI solution, previously heated at an initial pH of 8.2, at 90°C for 8 h was significantly lower ( $P \leq 0.05$ ) than that of the corresponding unheated WPI solution in the pH range 3.0-5.5, with the lowest protein solubility (24.9%) at pH 4.0 for the heated WPI solution, compared to a solubility of 88.0% for the unheated WPI solution at the same pH. The decrease in solubility of whey protein caused by heating is attributed to either unfolding of protein structure, which exposes hydrophobic/sulfhydryl reactive sites, resulting in greater protein-protein interactions or heat-induced crosslinking of the protein molecules (Mulcahy *et al.* 2016a). In the pH range 3.0-5.0, the WPI-MD17 solution that was heated for 8 h at 90°C had significantly higher ( $P \leq 0.05$ ) protein solubility than the WPI solution heated without maltodextrin. The greatest improvement was observed at pH 3.5, where the heated WPI solution had a protein solubility of just 26.7%, compared to the heated WPI-MD17 solution, which had a protein solubility of 50.7%, while both the heated WPI and WPI-MD17 had lowest protein solubility of 24.9 and 30.1%, respectively, at pH 4.0.

Unheated WPH and WPH-MD17 solutions had a protein solubility of 81.1% at pH 4.0-4.5 which was lower than the protein solubility of the unheated WPI in the same pH range (Fig. 6.5B). This could be attributed to the exposure of buried hydrophobic residues and/or release of specific peptides during hydrolysis that promote peptide/protein-peptide aggregation (Creusot & Gruppen, 2007b). The

heated WPH solution (8 h at 90°C) had a similar protein solubility across the pH range 2.0-8.0 to the unheated WPH solution. Peptides produced by enzymatic hydrolysis have smaller molecular sizes and less secondary structure than intact proteins which can limit heat-induced changes (Chobert *et al.*, 1988). At pH 4.0-4.5, the protein solubility of the heated WPH-MD17 solution increased significantly ( $P \leq 0.05$ ) by 5.6% compared to that of the heated WPH solution (75.3% protein solubility). This increase in protein solubility of solutions of the heated WPH-MD17 conjugate, compared to solutions of WPH heated (8 h at 90°C) without MD17, and solutions of WPI conjugated with MD17 (8 h at 90°C), can be attributed to enhanced hydration of the protein due to steric hindrance between the protein molecules provided by the attachment of the bulky dextran molecules (Wang & Ismail, 2013).

#### 6.4.8 *Changes in physicochemical properties and microstructure on heating of protein solutions containing added NaCl*

Confocal laser scanning microscopy (CLSM) analysis showed that small protein aggregates ( $< \sim 1 \mu\text{m}$ ) were present in the unheated WPI and WPH solutions (Fig. 6.6 1A and 4A) and no differences were observed between the unheated WPI/WPH solutions and the WPI/WPH solutions containing MD17 (data not shown). Once the previously unheated WPI solution (with 40 mM NaCl added subsequently) was heated at 85°C, at pH 6.8, for 10 min, the protein formed a turbid, continuous gel network (Fig. 6.6 1B).



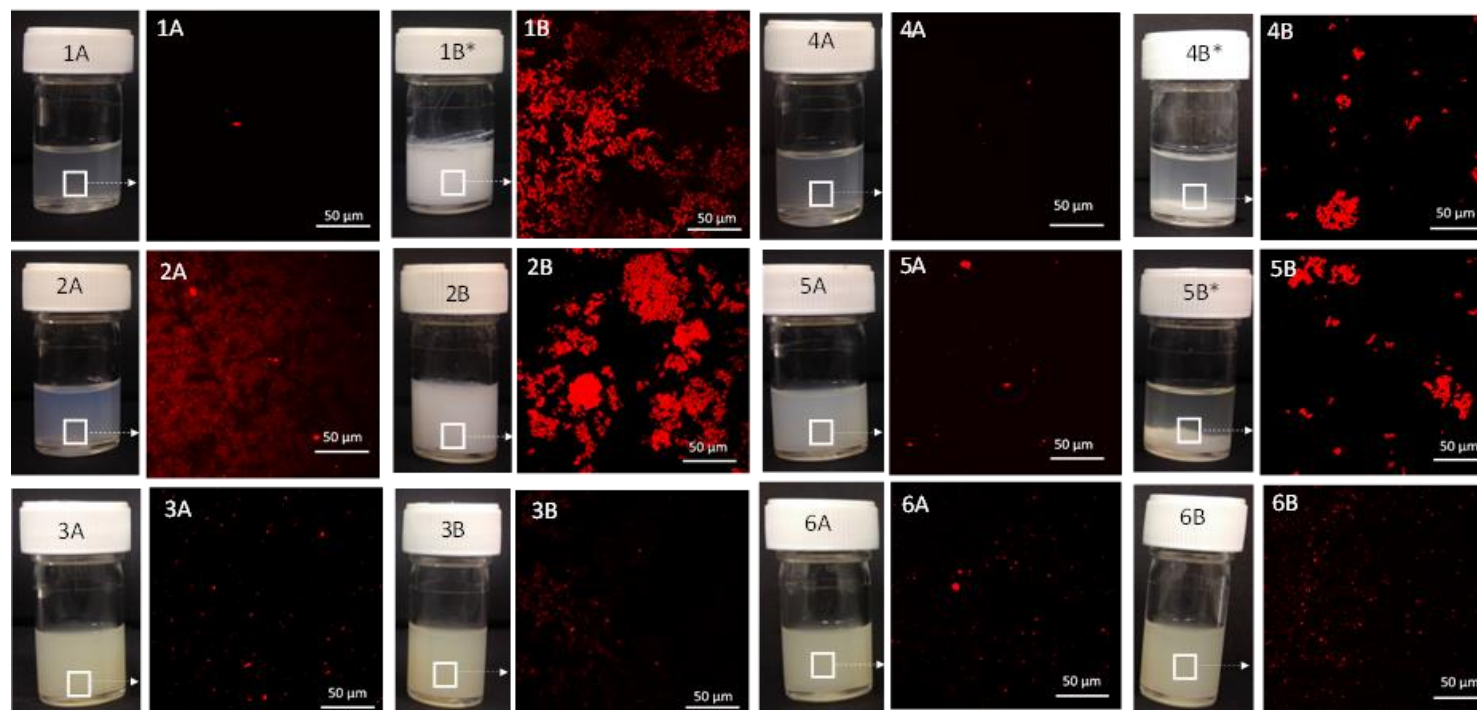
**Fig. 6.5.** Protein solubility as a function of pH for whey protein isolate (WPI), whey protein hydrolysate (WPH) and WPI/WPH-maltodextrin (MD17) solutions unheated (---) or heated (—) at an initial pH of 8.2 at 90°C for 8 h; (A) WPI (○ or ●) and WPI-MD17 (□ or ■); (B) WPH (◇ or ◆) and WPH-MD17 (Δ or ▲)

The presence of NaCl in protein solutions can decrease the inter-protein charge repulsions which can lead to increased protein-protein interactions, protein aggregation and gel formation (Ryan *et al.*, 2012). The WPI solution that was heated for 8 h at 90°C showed an increase in the number of small dense protein aggregates ( $<1\ \mu\text{m}$ ) when compared to the unheated WPI solution (Fig. 6.6 2A).

It is clear from these results that covalent attachment of the bulky hydrophilic MD to whey protein molecules through Maillard-induced conjugation greatly enhanced stability of whey protein/peptide solutions when subsequently thermally treated in the presence of NaCl. The improvement in thermal stability of whey protein following conjugation with MD is most likely due to enhanced steric hindrance due to the attachment of carbohydrate side chains helping to prevent further heat-induced destabilisation, even in the presence of NaCl. This previously heated WPI solution (8 h at 90°C) increased in turbidity by 56.4% compared to the pre-heated control, and large protein aggregates ( $\sim 30\text{--}50\ \mu\text{m}$ ) formed but the solution did not gel when it subsequently had 40 mM NaCl added and received a further heat treatment (10 min at 85°C; Fig. 6.6 2B). This indicated that pre-heated intact whey proteins were less susceptible to further heat-induced structural changes and gelation compared to untreated whey proteins on further heat treatment in the presence of added salt (Fig 5.6 1B).

The protein/peptides in the unheated and previously heated WPH solution precipitated when further heated with added NaCl and large protein aggregates formed ( $\sim 10\text{--}50\ \mu\text{m}$ ; Fig 5.6 4B and 5B); turbidity was not measured in the WPH solutions which showed precipitation. Precipitation of the proteins/peptides in those WPH solutions was likely due to the exposure of hydrophobic groups during

enzymatic hydrolysis of the protein or the presence of more ionic groups in the WPH solutions due to base addition for neutralisation during production of the hydrolysed ingredient compared to the WPI (Adler-Nissen, 1986). Solutions of WPI-MD17 or WPH-MD17 conjugates (i.e., previously heated for 8 h at 90°C at an initial pH of 8.2) which had 40 mM NaCl added, and were subsequently heated at 85°C for 10 min, increased in turbidity by just 2.1% and 0.5% compared to their respective controls, and the protein aggregates present were small ( $< \sim 1 \mu\text{m}$ ; Fig 5.6. 3B and 6B).



*Fig. 6.6. Photographs and confocal laser scanning microscopy images of (1 and 2) WPI, (3) WPI-MD17, (4 and 5) WPH and (6) WPH-MD17. The solutions contained 2.5% protein, at pH 6.8, before (A) and after (B) heating at 85°C for 10 min; all solutions contained 40 mM added NaCl. Photographs labelled with 1 and 4 received no prior heat treatment and 2, 3, 5 and 6 were previously heated at 90°C for 8 h at an initial pH of 8.2; solutions labelled (A) are the respective controls for the sample set and solutions marked with \* gelled/precipitated. Micrographs present distribution of protein (red) in different solutions. Scale bar (bottom right) = 50 μm.*

## 6.5 Conclusion

This study demonstrated that the physical state of whey protein molecules (i.e., intact or hydrolysed) had a considerable impact on the rate and extent of protein-carbohydrate conjugation. Conjugation of intact and hydrolysed whey proteins with maltodextrin was achieved by heating solutions at an initial pH 8.2, at 90°C for 8 h. Low levels of hydrolysis of the whey protein molecules resulted in an increase in the rate and extent of conjugation of the whey protein with maltodextrin, with limited associated development of colour and advanced Maillard reaction products. Intact and hydrolysed whey protein-maltodextrin conjugate solutions had significantly ( $P \leq 0.05$ ) increased protein solubility in the pH range 4-5 and the thermal stability (in an added salt environment) was superior to those of the unheated or heated control solutions. Conjugation with maltodextrin provides a potential method to enhance the functionality of hydrolysed whey proteins in food applications. Further investigation of such protein-carbohydrate conjugates in formulated food applications is required.

## 6.6 Acknowledgements

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## **Chapter 7**

*Overall conclusions & suggestions for future research*

## 7.1 Overall conclusions

A rapidly-growing application for whey protein (particularly whey protein isolate; WPI) ingredients is protein-based beverages (Cochrane *et al.*, 2012). Arising from thermal treatment, required to extend shelf-life, together with the formulation complexity associated with nutritional beverages, limitations such as turbidity development, protein coagulation, sedimentation and even gelation, are often experienced on incorporation of WPI ingredients in nutritional beverages (LaClair & Etzel, 2010). However, consumer acceptance of nutritional beverages is based on clarity of these beverage solutions, with consumers rating clear beverages as having superior sensory and ‘thirst-quenching’ attributes compared with opaque beverages (Beucler *et al.*, 2005).

Maillard-induced conjugation of whey proteins/peptides under controlled conditions offers considerable potential in the development of whey protein-based ingredients with enhanced heat stability and solubility. The improvement in the functional properties (e.g., solubility and emulsification) of whey protein/peptide-carbohydrate conjugates under both wet and dry heating conditions has been attributed to several mechanisms including changes in conformation of proteins arising from conjugation (i.e., unfolding of the protein structure and exposure of hydrophobic and hydrophilic groups) and increased steric stabilisation provided by the covalent attachment of the carbohydrate moieties to the protein/peptide molecules. It is essential to establish the relationship between the extent of protein-carbohydrate conjugation and the functional properties of the resultant protein-carbohydrate systems.



The objectives of the research reported in this thesis were to develop, and comprehensively characterise, the functional properties of whey protein-carbohydrate conjugates. The studies described within have facilitated comprehensive, novel investigations into the impact of the physicochemical properties of whey proteins and carbohydrates and mode of conjugation (i.e., wet and dry heating) on the functionality of Maillard reaction-induced protein-carbohydrate conjugates. Some of the key findings from the work completed as part of this PhD project may be summarised as follows.

In the first part of this thesis the influence of starch hydrolysis product chain-length on the rate and extent of Maillard-induced conjugation of whey proteins under wet heating conditions was studied (**Chapter 2**). The reactivity of a range of starch hydrolysis products with different dextrose equivalent (DE) values (i.e., maltodextrin, MD; corn syrup solids, CSS, and glucose) during conjugation with whey proteins was determined. The rate and extent of conjugation of whey proteins increased on heating for up to 8 h with decreasing DE value of the starch hydrolysis products from MD (DE 6, 12 and 17), through CSS (DE 30 and 38) to glucose. This was attributed to an increase in the number of free carbonyl groups per unit weight of the carbohydrate, with increasing DE value.

The extent of protein-carbohydrate conjugation was optimised after 8 h of wet heating as high levels of conjugation were achieved with limited associated progress of the Maillard reaction to the advanced stages.

The wet heating conditions used to achieve whey protein-carbohydrate conjugation were also conducive to forming covalent cross-links (e.g., lysinoalanine; LAL) between protein molecules due to heating and exposure to moderate alkaline

conditions. However, conjugation of whey protein molecules with starch hydrolysis products resulted in smaller particles forming and lower levels of LAL compared to the heated whey protein controls, indicating that conjugation may limit inter-/intra-molecular protein crosslinking and aggregation (**Chapter 2**). Such changes in protein aggregation/crosslinking, combined with steric hindrance provided by the attachment of bulky, hydrophilic carbohydrate molecules caused alteration of the functional properties of the whey protein-carbohydrate conjugates, including increased solubility and thermal stability of the whey protein conjugates relative to the control unconjugated solutions (**Chapter 3**). The DE value of starch hydrolysis products attached to the whey protein molecules affected their solubility and thermal stability; solutions of conjugated WPI-MD38 and WPI-MD6 heated for 8 h had up to 32% increased protein solubility and maintained solution clarity on heating compared with heated WPI controls.

Dry heating facilitated conjugation of maltodextrin to whey protein molecules, while restricting whey protein denaturation and limiting the progression of the Maillard reaction to advanced stages (**Chapter 4**). Dry heating of WPI and MD6 for 24 h achieved a level of conjugation similar to the WPI-MD6 conjugate prepared by wet heating for 8 h. However, conjugation of WPI using dry heating yielded greater levels of native whey protein compared to wet heating which impacted the resulting functionalities of the dry heated WPI-MD6. Whey protein solutions prepared from dry heated WPI-MD6 conjugates had the highest solubility across the pH range 2-8, compared to all unheated and heated controls. Conjugation of WPI-MD6 by dry heating for 24 h also improved thermal stability and maintained solution clarity in an added salt environment, compared to the respective unheated

and dry-heated WPI control samples. These results showed that dry heating of WPI with MD could be used as a potential tool to enhance solubility and thermal stability in products where the retention of the native state of whey protein is considered advantageous.

There is an increasing global demand for hydrolysed whey protein ingredients; however, hydrolysis of whey proteins often results in impairment of their functional properties due to the exposure of previously-buried hydrophobic residues and/or release of specific peptides that promote peptide–peptide and peptide-protein aggregation. However, hydrolysis of whey proteins may render additional  $\alpha$ -amino groups available and expose previously-buried lysyl residues within the whey protein material used for conjugation, due to disruption of protein structure (**Chapter 5**). The total number of amino groups available to react with the carbonyl groups of reducing carbohydrates was 55.4% higher in the whey protein hydrolysate (WPH) solution than in the WPI solution, conferring on the WPH a greater propensity to undergo Maillard-induced changes during heating. Conjugation of intact and hydrolysed whey proteins with MD17 was achieved by wet heating and a low level of hydrolysis (degree of hydrolysis of 9.3%) of whey proteins resulted in an increase in the rate and extent of conjugation with maltodextrin, with limited associated development of colour and advanced Maillard products.

Intact and hydrolysed whey protein-MD17 conjugate solutions had increased protein solubility (5.0-24.1%) in the pH range 4.0-5.0 and the thermal stability (in an added salt environment) was greater than those of the unheated or heated control solutions. Conjugation of hydrolysed whey proteins with MD17 was shown to be a promising method for extending the functional properties, in particular thermal

stability, of hydrolysed whey protein ingredients. A complementary study completed by Drapala *et al.* (2016a) reported that model infant formula emulsions (1.5% protein) prepared with conjugated WPH-MD12 demonstrated excellent thermal stability, with no changes in fat globule size distribution or microstructure on heat treatment at 100°C for 15 min due principally to increased steric stabilisation as a result of conjugation.

The influence of aggregation state on availability of amino groups in whey proteins was investigated to gain a better understanding of the ability of whey proteins to undergo conjugation with carbohydrates (**Chapter 6**). Varying extents of aggregation of whey proteins were achieved by modifying the compositional and environmental conditions of the whey protein solutions; salt was added to promote aggregation of the whey protein molecules, whereas a reducing agent was added to inhibit the formation of disulfide and sulfhydryl-disulfide-mediated whey protein aggregates. Heat- and salt-induced aggregation of whey proteins impacted the availability of amino groups as measured by the OPA and TNBS methods. The OPA method for measuring available amino groups was less impacted by heat-induced structural changes of whey protein molecules, in particular, sulfhydryl-disulfide interchange and disulphide-bond mediated aggregation than the TNBS method. This was attributed to the amino groups being located within large, dense protein aggregates, hence becoming unavailable for reaction with the TNBS molecules. These findings may have implications for those measuring the consumption or liberation of available amino groups during protein-carbohydrate conjugation or protein hydrolysis, respectively.

In summary, the studies reported in this thesis have generated new insights into controlled conjugation of whey proteins/peptides with starch hydrolysis products specifically in terms of optimising their preparation and studying their functional characteristics to facilitate their incorporation into food formulations. Such outputs provide a better understanding of how Maillard-induced conjugation may be applied industrially in order to maximise new techno-functional outputs from whey protein ingredients.

## **7.2 Suggestions for future research**

Follow-up studies that would be complimentary to the work presented in this thesis include:

### **Targeted approach to hydrolysis of whey protein substrates for conjugation**

Heat-induced denaturation and aggregation of whey proteins involves a complex number of reactions, with  $\beta$ -lg dominating the denaturation and aggregation process due to its higher concentration and greater reactivity compared to other whey proteins. Aggregation of whey proteins in this manner can decrease the availability of amino groups, leading to a decreased propensity of protein molecules to undergo conjugation (**Chapter 6**). A targeted approach to hydrolysis of individual whey proteins (e.g., preferentially hydrolyse  $\beta$ -lg) by using specific enzymes and time-temperature combinations (e.g., chymotrypsin, 25°C, pH 8.5) (Lisak *et al.*, 2013) may allow more control over the availability of amino groups within heated whey

protein/peptide solutions, thereby increasing the rate of protein-carbohydrate conjugation.

### **Extended functionality of hydrolysed whey protein ingredients**

Low- to moderately-hydrolysed whey proteins are used in easy-to-digest infant formulas designed for infants that suffer from difficulty digesting intact proteins and also in sports nutritional products (Nguyen *et al.*, 2015). Good solubility, emulsification and thermal stability are important attributes of these protein ingredients and determine rheological/structural behaviour within a formulation; however, hydrolysis of protein can lead to impairment of these functional properties due to exposure of previously-buried hydrophobic regions and increased propensity for the hydrolysed whey proteins to aggregate (Drapala *et al.*, 2016a, and b). A targeted approach to protein hydrolysis in combination with conjugation of the resulting proteins/peptides with carbohydrates may also allow for expanded functionalities (e.g., high thermal stability) of whey protein hydrolysates in formulated food systems.

### **Hypoallergenic whey proteins with improved sensory attributes**

The incorporation of extensively hydrolysed hypoallergenic whey protein hydrolysates into nutritional formulations is required to provide specialised support for those with particular physiological needs (e.g., milk protein allergies) (Clemente, 2000; Nnanna & Wu, 2007). However, extensively hydrolysed whey proteins are associated with very poor sensory attributes, such as extreme bitterness, due to

release of hydrophobic amino acids and peptides (Newman *et al.*, 2015). Böttger *et al.* (2013), Zhang *et al.* (2014) and Zhong *et al.* (2014) reported significant reductions in the allergenicity of intact WPI,  $\alpha$ -lac and  $\beta$ -lg, respectively, when conjugated to carbohydrates under wet and dry heating conditions. Limited hydrolysis of the allergenic proteins (i.e., instead of extensively hydrolysed protein) in combination with conjugation of the proteins with carbohydrates may have the potential to mask the immunogenic epitope sites. The covalent attachment of hydrophilic carbohydrate moieties to peptides during conjugation in combination with moderate degrees of hydrolysis of the proteins may improve the overall sensory acceptability of hypoallergenic whey proteins.

### **Determine optimal whey protein substrate for conjugation**

Further scope exists to determine the optimum whey protein substrate for conjugation; conjugates (WPI-MD17) prepared from WPI produced by ion exchange chromatography (**Chapter 2**) had a 10% greater reduction in AAG compared to those prepared from membrane filtration-derived WPI (**Chapter 5**) after the same thermal treatment (initial pH 8.2, at 90°C for 8 h). WPI produced by membrane filtration from sweet whey contains ~20% glycomacropeptide, of which up to ~50% of the CMP is already glycosylated and unavailable to participate in the Maillard reaction (Kreuss *et al.*, 2009). Essentially, solutions prepared from WPI produced by membrane filtration, on a constant protein basis, effectively have a diluted level of protein material that can be conjugated with carbohydrates due to the presence of CMP. Other considerations such as the source of whey (cheese *vs* acid whey) and the fractionation and enrichment technology for manufacturing WPI can change the

mineral content (e.g., calcium) and relative proportions of individual whey proteins and affect whey protein conformation, which should be considered when choosing a whey protein substrate for conjugation.

### **Use of protein-carbohydrate conjugates as antioxidants in formulated foods**

Advanced Maillard products are largely responsible for some of the less desirable aspects of the Maillard reaction (e.g., off-flavours, loss of nutritional value, protein crosslinking and generation of potentially toxic compounds). However, Maillard reaction products in conjugated milk protein-carbohydrate systems have been associated with high levels of antioxidant activity as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals can be scavenged by Maillard reaction products through donation of hydrogen ( $H^+$ ) to form stable DPPH-H (Matthaus, 2002; Jiang & Brodkorb, 2012).

Advanced Maillard reaction products (e.g., melanoidins, pronyl-lysine) have been reported to exert structure-specific antioxidant activity and to delay, or inhibit carcinogenesis activity in a cell culture model with human intestinal cells (Lindenmeier *et al.*, 2002). Rufián-Henares and Morales (2007) reported that coffee melanoidins exert an iron chelation-mediated bacteriostatic activity and angiotensin-I converting enzyme (ACE) inhibitory activity which was significantly higher in coffee beans that had been heated for prolonged periods of time. As whey protein/peptide-maltodextrin conjugates have been shown to improve emulsion formation properties, processing and shelf life stability (Drapala *et al.*, 2016a,b), the use of whey protein/peptide-carbohydrate conjugates may also be of interest as



potential antioxidants in oil/water (e.g., infant formula emulsions, coffee creamers, mayonnaise) or water/oil emulsions (e.g., vegetable oil or dairy spreads).

### **Commercial optimisation of whey protein-carbohydrate conjugate production**

The results presented in this thesis (**Chapters 2, 3 and 5**) indicated that whey protein-carbohydrate conjugates had improved functional properties compared to their unconjugated counterparts. A complimentary study completed in parallel by Drapala *et al.*, 2016a and b demonstrated that model infant formula emulsions stabilised by WPH-MD conjugates (produced by wet heating at an initial pH 8.2, 90°C, for 8 h) had greater thermal (95°C, 15 min) and accelerated storage stability (40°C, 10 d) than emulsions stabilised by WPH + CITREM (9 g L<sup>-1</sup>) or WPH + lecithin (9 g L<sup>-1</sup>). The improvement in the stability of the model infant formula emulsions stabilised by the WPH-MD conjugate was attributed to the resistance to heat-induced bridging flocculation, compared to those stabilised by non-conjugated WPH. Further commercial scope exists to determine if whey protein-carbohydrate solutions prepared at <8 h heating had similar improvements in functionality as those detailed by Drapala *et al.* (2016a,b).

### **Nutritional and toxicological properties of conjugated proteins**

A number of authors have reported that conjugation of whey proteins with carbohydrates may alter the digestibility of the proteins. This altered nutritional profile is dependent on the heat treatment applied, exact conformational changes to the proteins and the molecular structure of the carbohydrate moiety covalently attached (Böttger *et al.*, 2013; Corzo-Martínez *et al.*, 2012). Further research on the

nutritional and toxicological aspects of milk protein/peptide-carbohydrate conjugates, and how conjugation impacts protein digestion and allergenicity, would help in the development of these ingredients for hypoallergenic and easy-to-digest food applications.

### **Novel methods to induce conjugation**

In addition to dry and wet heating approaches, some alternative technologies and combinations thereof, such as sonication, micro-fluidisation, high hydrostatic pressure and microwave heating are studied increasingly for application in the pre-treatment of whey proteins prior to conjugation or directly in the production of conjugates and may offer promise for the generation of whey protein/peptide conjugates with modified functionality. In particular, sonication is an under-utilised technology that may be used to induce protein-carbohydrate conjugation. At low frequencies (20–100 kHz), and higher power levels, ultrasound generates acoustic cavitation where micro-bubbles that are present in the solution grow in size until a critical size is reached, that implode, generating localised temperature hot spots and pressures of several thousand bar (Suslick *et al.*, 1999). Unique physical, mechanical or chemical effects of high-intensity ultrasonic waves are capable of altering protein properties through generation of immense pressure, shear stresses, turbulence, dynamic agitation, and temperature gradient in the medium through which they propagate (Knorr *et al.*, 2004). For example, high-intensity sonication can modify secondary structure of  $\beta$ -lg leading to unfolding of the protein molecules and increased covalent attachment of carbohydrates (e.g., ribose) at low temperatures

(10-15°C) with minor alterations in secondary and tertiary structures of the protein (Perusko *et al.* (2015)).

### **Fractionation technologies to enrich/purify conjugates**

The market for sports nutritional products is growing rapidly with low carbohydrate (<10% carbohydrate) sports beverages being more popular than their high carbohydrate counterparts (Martínez-Lagunas *et al.*, 2010). Consumers have rated clear sports beverages as having superior acceptability compared to opaque beverages (Beucler *et al.*, 2005). Protein/peptide-carbohydrate conjugates have been shown to help maintain whey protein solution clarity on heating in high ionic strength environments (**Chapters 2 and 5**); however, excess unconjugated carbohydrate may not be desirable in such applications. Several authors have purified protein-carbohydrate conjugates by chromatographic techniques (Aminlari *et al.*, 2005; Bund *et al.*, 2012; Gu *et al.*, 2010; Zhu *et al.*, 2010). Further scope exists to determine if any unreacted carbohydrate can be removed from the conjugated whey protein-carbohydrate systems by membrane filtration, e.g., ultrafiltration, in order to enrich or purify protein-carbohydrate conjugates for application in low-carbohydrate nutritional products (e.g., sports nutrition products).

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## **Appendix**

Publications in international peer-reviewed journals from this PhD project:

**Mulcahy, E. M.**, Fargier-Lagrange, M., Mulvihill, D. M., & O'Mahony, J. A. (2017). Characterisation of heat-induced protein aggregation in whey protein isolate and the influence of aggregation on the availability of amino groups as measured by the o-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods. *Food Chemistry*, 229, 66-74. (Based on Chapter 2).

**Mulcahy, E. M.**, Mulvihill, D. M., & O'Mahony, J. A. (2016). Physicochemical properties of whey protein conjugated with starch hydrolysis products of different dextrose equivalent values. *International Dairy Journal*, 53, 20-28. (Based on Chapters 3 and 4)

**Mulcahy, E. M.**, Park, C. W., Drake, M., Mulvihill, D. M., & O'Mahony, J. A. (2017). Enhancement of the functional properties of whey protein by conjugation with maltodextrin under dry heating conditions. *International Journal of Dairy Technology* (Accepted, based on Chapter 5).

**Mulcahy, E. M.**, Park, C. W., Drake, M., Mulvihill, D. M., & O'Mahony, J. A. (2016). Improvement of the functional properties of whey protein hydrolysate by conjugation with maltodextrin. *International Dairy Journal*, 60, 47-54. (Based on Chapter 6).

O'Mahony, J. A., Drapala, K. P., **Mulcahy, E. M.**, & Mulvihill, D. M. (2016). Controlled glycation of milk proteins and peptides: Functional properties. *International Dairy Journal*. [In press, based, in part, on Chapter 1)