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

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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. WPI-MD and WPH-MD solutions heated for 8 h had significantly higher ($P < 0.05$) 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.

1. Introduction

The Maillard reaction was first described by the French biochemist, Louis-Camille Maillard, in 1912 and is initiated when the ϵ -amino groups of lysyl residues and the α -amino groups of terminal amino acids in proteins condense with the carbonyl groups of reducing carbohydrates (Martins, Jongen, & Van Boekel, 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, Ru, & Ding, 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, Joye, Espinal-Ruiz, & McClements, 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, Davis, Doucet, & McGuffey, 2002). Controlled hydrolysis of whey proteins alters the physicochemical functionalities, including

the solubility, heat stability, viscosity, emulsifying and foaming properties (Singh & Dalgleish, 1998; Wijayanti, Bansal, & Deeth, 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, Otte, Madsen, and Qvist (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, Doran, Torley, & Agboola, 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).

2. Material and methods

2.1. 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, 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, Ward, Froehner, Brody, & Schroeder, 2002). 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, Edwards, and Woollard (1996) with minor modifications as detailed by Mulcahy, Mulvihill, and O'Mahony (2016). 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.

2.2. *Size exclusion chromatography*

Size exclusion chromatography (SEC) was carried out as described by O'Loughlin et al. (2013) to determine the molecular mass distribution profile of the proteins and peptides in the WPI and WPH.

2.3. *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. (2016); 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.

2.4. *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 ortho-phthalaldehyde (OPA) method as described by

Nielsen, Petersen, and Dambmann (2001) with minor modifications as described by Mulcahy et al. (2016).

2.5 *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). LAL was extracted as described by Calabrese, Mamone, Caira, Ferranti, and Addeo (2009) with minor modifications. Samples were not derivatised prior to analysis and the acid hydrolysate was diluted 1:10 with HPLC grade water. Injections were made onto a Waters Acquity HSS T3 C18 column (1.7 μm , 2.1×100 mm) at a flow rate of 0.5 mL min^{-1} and maintained at 40°C . The isocratic mobile phase consisted of 0.1% formic acid in water. Detection was performed with 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^{-1} and LAL was quantified using the monovalent charged ion 234.04 in selective ion monitoring mode. An 8 point standard curve was constructed ranging from $25 \mu\text{g L}^{-1}$ to 5 mg L^{-1} for quantification.

2.6 *Fluorescence of advanced Maillard products*

The fluorescence of advanced Maillard products (F_{AMP}) was measured as described by Mulcahy et al. (2016). 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 products (F_{AMP}) was expressed as relative fluorescence units per gram of protein ($RFU\ g^{-1}$ protein).

2.7. *Measurement of colour*

The colour of the unheated and heated WPI/WPH and WPI/WPH-MD17 solutions (5%, w/v, protein) was determined as described by Mulcahy et al. (2016) using a pre-calibrated colorimeter (Minolta Chroma Meter CR-400, Minolta Ltd., Milton Keynes, UK). Colour was expressed as the Commission Internationale de l'Eclairage (CIE) L^* colour chromaticity coordinate.

2.8. *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. (2016).

2.9. *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. (2016). 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 (treated in the same manner to 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, Auty, Mulvihill, and O'Mahony (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.

2.10. 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 Duncan'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 $P < 0.05$.

3. Results and Discussion

3.1. 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 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 protein had a molecular mass > 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 1). The majority (50.5%) of protein in the WPI had a molecular mass in the range 10–20 kDa; a range that represents the major native whey protein fractions β -lactoglobulin (β -lg; 18.4 kDa) and α -lactalbumin (α -lac, 14.2 kDa). The remaining material (21.5% of protein) had a molecular mass between 0–10 kDa, which is likely due to the presence of glycomacropeptide (~7.5 kDa; Sharma, Rajput & Mann, 2013), small amounts of proteose peptones or other low molecular mass protein components (Saint-Sauveur, Gauthier, Boutin, & Montoni, 2008).

The WPH had 12.0% of protein with molecular mass > 20 kDa that could be attributed to the low abundance of high molecular mass 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 protein in the

molecular mass range 10–20 kDa corresponding mainly to residual unhydrolysed β -lg and α -lac. The remaining material (61.9%) had a molecular mass range of 0–10 kDa that may be associated with peptides and amino acids. A small amount (1.8%) of insoluble material was present in the WPH, which is likely due to the formation of high molecular mass 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).

3.2. *Change in available amino groups on heating*

Only the α -amino group of a terminal amino acid or the ϵ -amino group of lysyl residues can partake in the Maillard reaction, which amounts to 16 and 13 potential available conjugation sites per β -lg and α -lac molecule, respectively (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 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 α -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. 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% 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 interaction between proteins and lactose, as the WPH contained more lactose than the WPI (Table 1) or 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).

The WPI/WPH solutions containing MD17 had the greatest reduction in AAG compared with 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% compared with a 26% reduction in AAG for hydrolysed WPH-MD17 solutions after the same treatment. The greatest reduction in AAG groups for WPH-MD17 solutions 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% reduction in AAG on conjugation of WPI-MD17 compared with a 21.1% reduction on conjugation of WPH-MD17.

3.3. Development of lysinoalanine on heating

The presence of lysinoalanine (LAL) in protein solutions has been reported to be responsible for protein crosslinking leading to impairment of functional properties and loss of nutritional value (Pellegrino, Van Boekel, Gruppen, Resmini, & Pagani, 1999). The WPI solution heated for 8 h at 90°C had a high level of LAL (178.6 mg 100 g⁻¹ protein) compared with the unheated WPI solution (14.1 mg 100 g⁻¹ protein). The level of LAL in the heated WPI-MD17 solution (58.8 mg 100 g⁻¹ protein) at 8 h was significantly ($P < 0.05$) lower than in the heated WPI solution. However, the level of LAL in the heated WPH solution (100.1 mg 100 g⁻¹ protein) at 8 h was not significantly ($P > 0.05$) different from that in the heated WPH-MD17 solution (98.2 mg 100 g⁻¹ protein) at 8 h. Heat treatment of proteins, especially at alkaline pH, can lead to the formation of LAL by β -elimination of seryl or cystyl residues to form the intermediate dehydroalanine, which can then react with the ϵ -amino group of

lysine (Calabrese et al., 2009). As the ϵ -amino group of lysine can also be consumed by the covalent attachment of maltodextrin to protein during the Maillard reaction (Fig. 1), competition for the ϵ -amino reaction sites is likely to result in lower levels of LAL in heated WPI/WPH-MD17 solutions compared with the heated WPI solution.

3.4. *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 minimise 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 ($P < 0.05$) from pH 8.2 to 7.4 on heating for 24 h (Fig. 2). The decrease in pH can be attributed to the production of organic acids (i.e., formic or acetic acid) during the intermediate 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 to be 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.

3.5. *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. 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 with the solutions at 24 h of heating; at heating times > 8 h, there were significant decreases ($P < 0.05$) in the L^* value for all solutions containing maltodextrin, with an accompanying increase in brown colour being evident.

3.6. Fluorescence of advanced Maillard products

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 products (F_{AMP}) was monitored throughout the 24 h heating time course (Fig. 4). All the unheated solutions of WPI/WPH and WPI/WPH-MD17 had low levels of F_{AMP} (2.4-3.5

relative fluorescence units; RFU) and the WPI solution did not significantly ($P > 0.05$) increase in F_{AMP} (3.7 RFU) after 24 h of heating at 90 °C.

There was relatively limited development of F_{AMP} in the WPI-MD17 solution (13.0 RFU) after heating for 24 h. The F_{AMP} increased linearly ($R^2 > 0.98$) as a function of heating time for the WPH and WPI/WPH-MD17 solutions. The level of F_{AMP} was significantly ($P < 0.05$) higher in both the WPH (36.1 RFU) and the WPH-MD17 (91.7 RFU) solutions when compared with 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 β -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_{AMP} in the solutions. The WPI/WPH-MD17 solutions after 8 h of heating which were found to have significantly ($P < 0.05$) lower levels of F_{AMP} (6.1 and 31.7 RFU) and colour development than those heated for 24 h and were thus chosen for assessment of functionality.

3.7. Protein solubility

Unheated WPI and WPI-MD17 solutions had high protein solubility ($> 88.0\%$) 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. 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 ($P < 0.05$) 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 with a solubility of 88% for the unheated WPI solution at the same pH. The decrease in solubility of whey protein after heating is attributed to either unfolding of protein structure, which exposes hydrophobic/sulphydryl reactive sites, resulting in greater protein-

protein interactions occurring or heat induced crosslinking of the protein molecules (Mulcahy et al., 2016). In the pH range 3.0–5.0, the WPI-MD17 solution that was heated for 8 h at 90 °C had significantly higher ($- < 0.05$) 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 with the heated WPI-MD17 solution that 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. 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 masses and less secondary structure than intact proteins, which can limit heat-induced changes (Chobert, Bertrand-Harb & Nicolas, 1988). At pH 4.0–4.5, the protein solubility of the heated WPH-MD17 solution increased significantly ($P < 0.05$) by 5.6% compared with that of the heated WPH solution (75.3% protein solubility). This increase in protein solubility of solutions of the heated WPH-MD17 conjugate compared with 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 the steric hindrance between the protein molecules provided by the attachment of the bulky dextran molecules.

3.8. Changes in physicochemical properties and microstructure after heating of protein solutions containing added NaCl

Confocal laser scanning microscopy (CLSM) showed that small protein aggregates ($< \sim 1 \mu\text{m}$) were present in the unheated WPI and WPH solutions (Fig. 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; 1B). 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 ($< \sim 1 \mu\text{m}$) when compared with the unheated WPI solution (Fig. 6; 2A). This previously heated WPI solution (8 h at 90 °C) increased in turbidity by 56.4% compared with the pre-heated control and large protein aggregates ($\sim 30\text{--}50 \mu\text{m}$) formed but the solution did not gel when it was subsequently had 40 mM NaCl added and received a further heat treatment (10 min at 85 °C; Fig. 6; 2B). This indicated that pre-heated intact whey proteins were less susceptible to further heat-induced structural changes and gelation compared with untreated whey proteins on further heat treatment in the presence of added salt (Fig 6; 1B). Similar results were reported by Ryan et al. (2012) who showed that soluble whey protein aggregates formed by heating WPI for 10 min at 90 °C had improved thermal stability when further heated (90 °C for 5 min) in solutions that contained 0–108 mM added NaCl due to their altered aggregate shape and higher charge, which resulted in final aggregates that were smaller and less dense than in previously untreated WPI.

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 6, 4B and 5B); turbidity was not measured in the WPH solutions that showed precipitation.

Precipitation of the proteins/peptides in those WPH solutions is 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 with 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 of pH 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 with their respective controls, and the protein aggregates present remained small ($< \sim 1 \mu\text{m}$; Fig 6. 3B and 6B). It is clear from these results that covalent attachment of the bulky hydrophilic MD to whey proteins 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.

4. Conclusion

This study showed 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 products. Intact and hydrolysed whey protein-maltodextrin conjugate solutions had significantly ($P < 0.05$) 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.

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554 **Table 1**

555 Composition and degree of hydrolysis of the whey protein isolate (WPI) and whey protein
556 hydrolysate (WPH). ^a

Composition	WPI	WPH
Protein (total)	87.2 ± 0.9	83.7 ± 0.5
Lactose	0.4 ± 0.02	2.8 ± 0.1
Ash	2.8 ± 0.1	2.9 ± 0.1
Degree of hydrolysis	-	9.3 ± 0.7
Insoluble protein	0.0 ± 0.0	1.8 ± 0.6
> 20 kDa	28.0 ± 3.4	12.0 ± 1.6
10-20 kDa	50.5 ± 3.7	24.2 ± 8.8
5-10 kDa	4.9 ± 0.2	9.5 ± 1.9
2-5 kDa	15.6 ± 0.3	11.9 ± 1.6
0-2 kDa	0.9 ± 0.1	40.5 ± 2.6

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565 ^a Values are means ± standard deviation of data from three independent trials. Values for
566 protein, lactose, ash are a percentage on a w/w basis; degree of hydrolysis is the number of
567 peptide bonds cleaved expressed as a percentage of the original number of peptide bonds;
568 values for insoluble protein and different molecular mass ranges are percentages of total
569 protein.

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Figure legends

Fig. 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; ○, WPH; ▲, WPI-MD17; ●, WPH-MD17. Values are presented as mean ± standard deviation of data from three independent trials.

Fig. 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; ○, WPH; ▲, WPI-MD17; ●, WPH-MD17. Values are presented as mean ± standard deviation of data from three independent trials.

Fig. 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

Fig. 4. Fluorescence of advanced Maillard 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; ○, WPH; ▲, WPI-MD17; ●, WPH-MD17. Values are presented as mean ± standard deviation of data from three independent trials.

Fig. 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 (△ or ▲). Values are presented as mean ± standard deviation of data from three independent trials.

Fig. 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.

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519 **Fig. 1.**

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523 **Fig. 2.**

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Fig. 3.

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532 **Fig. 4.**

Fig. 5A.

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538 **Fig. 5B.**

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550 **Fig. 6.**

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515 **Fig. 3.**

Fig. 6