

Title	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 ortho-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods
Authors	Mulcahy, Eve M.;Fargier-Lagrange, Maéva;Mulvihill, Daniel M.;O'Mahony, James A.
Publication date	2017-02-02
Original Citation	Mulcahy, E. M., Fargier-Lagrange, M., Mulvihill, D. M. and 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 ortho-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods', Food Chemistry, 229, pp. 66-74. doi:10.1016/ j.foodchem.2017.01.155
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.foodchem.2017.01.155
Rights	© 2017 Elsevier B.V. All rights reserved. This manuscript version is made available under the CC-BY-NC-ND 4.0 license https://creativecommons.org/licenses/by/4.0/
Download date	2025-05-09 18:43:42
Item downloaded from	https://hdl.handle.net/10468/4467



University College Cork, Ireland Coláiste na hOllscoile Corcaigh

Accepted Manuscript

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 *ortho*-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods

Eve M. Mulcahy, Maéva Fargier-Lagrange, Daniel M. Mulvihill, James A. O'Mahony

PII:	S0308-8146(17)30168-1
DOI:	http://dx.doi.org/10.1016/j.foodchem.2017.01.155
Reference:	FOCH 20547
To appear in:	Food Chemistry
Received Date:	29 July 2016
Revised Date:	10 January 2017
Accepted Date:	31 January 2017

IST ON A HE
Autobile where a second according to an SciVerse ScienceDirect

Please cite this article as: Mulcahy, E.M., Fargier-Lagrange, M., Mulvihill, D.M., O'Mahony, J.A., 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 *ortho*-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods, *Food Chemistry* (2017), doi: http://dx.doi.org/10.1016/j.foodchem.2017.01.155

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 *ortho*-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods

Eve M. Mulcahy, Maéva Fargier-Lagrange, Daniel M. Mulvihill and James A. O'Mahony*

School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

*Corresponding author. Tel.: +353 21 4903625

E-mail address: sa.omahony@ucc.ie; (J. A. O'Mahony)

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 more covalent interactions between proteins, than the heated WPI+NEM and WPI+NaCl+NEM solutions. There were marked differences in the levels 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 by the OPA method. These results demonstrate that the

measurement of available amino groups by the OPA method is less impacted than by the TNBS method after heat-induced structural changes, arising from disulfide or sulfhydryldisulfide bond-mediated aggregation of whey protein molecules.

1. Introduction

It has been recognized, for over 100 years, that the ε -amino group of lysine and α 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 has triggered the development of various assays to study accessibility/availability of amino groups of proteins; the ortho-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods have been shown to be both rapid and sensitive for quantifying AAG in protein solutions (Rutherfurd, 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. 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 wavelength of 340 nm (Adler-Nissen, 1979; Fig. 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 residues of proteins,

the presence of insoluble protein material and heat treatment of proteins (Spellman, McEvoy, O'Cuinn & FitzGerald, 2003; Rutherfurd, 2010a).

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 proteinbased ingredients as it can impact both the bio-availability and techno-functional properties of the proteins (Cattaneo, Masotti & Pellegrino, 2009; Rutherfurd, 2010b; Rutherfurd & Moughan, 2005). The ε-amino group of lysine and α-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, & Brodkorb, 2012; Hurrell & Carpenter, 1981; Mehta & Deeth, 2015; Mulcahy, Mulvihill & O'Mahony, 2016).

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, Barbut, McGauley, Marcone & Narine, 2000; Oldfield, Singh & Taylor, 2005; Ryan, Zhong & Foegeding, 2013; Sağlam, Venema, de Vries & van der Linden, 2014). Fitzsimons, Mulvihill, and Morris (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, and 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 sulphydryl group interaction, disulphide interchanges (Sawyer, 1968), along with the contribution of non-covalent hydrophobic and/or electrostatic interactions (Hoffmann & van Mil, 1997; Verheul, Roefs, & de Kruif, 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 the mechanism of aggregation (Vardhanabhuti et al., 2001). The presence of ions (e.g., Na⁺, Ca²⁺), at sufficient concentrations in whey protein solutions, can result in intermolecular electrostatic shielding of negativelycharged 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; Verheul, Roefs, & de Kruif, 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).

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, Bouhallab, Mollé, O'Kennedy & Mehra, 2003). Hoffmann and van Mil (1997) reported that the presence of NEM during heating of β -lg solutions (1-5% protein, pH 7) prevents the formation of β -lg polymers on heating at 65°C for up to 48 h. Kitabatake, Wada and Fujita (2001) reported that, although disulphide interchanges were limited in a β -lactoglobulin (β -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). Mulcahy *et al.* (2016) reported that the AAG decreased by 4.8% in a whey protein isolate (WPI) solution heated at 90°C for 24 h (as measured by the OPA method), which was most likely due to structural rearrangements within the protein molecules. Solutions of β -lactoglobulin (β -lg) heated at 60°C for 72 h had 5.0% less AAG than had the unheated control when measured by the OPA method (Chevalier, Chobert, Popineau, Nicolasa, & Haertlé, 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 β -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 systematically determine the influence, if any, of heatinduced 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 extents 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.

Jook Calif

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI), BiPro[®], 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%) contents of the WPI were determined, using standard analytical procedures, as detailed by Mulcahy *et al.* (2016). Sodium (747 mg 100 g⁻¹ powder), calcium (70 mg 100 g⁻¹ powder), phosphorus (58 mg 100 g⁻¹ powder), potassium (29 mg 100 g⁻¹ powder) and magnesium (4.7 mg 100 g⁻¹ powder) contents in the WPI were measured, using inductively-coupled plasma-mass spectrometry, as described by Herwig, Stephan, Panne, Pritzkow and Vogl (2011). All chemicals were of analytical grade and were sourced from Sigma–Aldrich (Tallaght, Dublin, Ireland) unless otherwise stated.

.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 of 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. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-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 modifications, as described by Mulcahy *et al.* (2016). Briefly, samples in a reducing buffer containing 100 mM dithiothreitol (Pierce, Rockford, IL, USA), were heated at 95°C for 5 min before being loaded (15 μ g 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 in the manufactures instructions.

2.4. 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, 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 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.

2.5. Determination of particle size distribution

Particle size distributions of the unheated and heated WPI solutions were determined, using dynamic light scattering with a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK), as described by Mulcahy *et al.* (2016) to evaluate how 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 mean 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.

2.6. 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.

JS CRIF

2.7. Determination of available amino groups

2.7.1. Ortho-phthalaldehyde method

Available amino groups (AAG) were quantified by the *ortho*-phthalaldehyde (OPA) method, as described by Nielsen, Petersen and Dambmann (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.

2.7.2. 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 sulfate (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.

2.8. Statistical data 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.

3. Results and discussion

3.1. 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, Mulvihill and Morris (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 βlactoglobulin (β -lg) and α -lactalbumin (α -lac) bands in non-reducing SDS-PAGE analysis of the heated WPI (Fig. 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).

3.2. 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 1) with similar results reported by Giroux, Houde and Britten (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 ($P \le 0.05$) 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 β -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 β -lg solution without added NEM.

3.3. Influence of heating on whey protein aggregation

3.3.1. Effect of heating on aggregation of WPI, as determined by sodium dodecyl sulfatepolyacrylamide 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 β -lg and α -lac, along with whey protein oligomers (Fig. 2A and B, lane 2). The heated WPI solution had less monomeric β -lg and α -lac (Fig. 2A, lane 3) resolved in the nonreducing SDS-PAGE gel than had the unheated WPI solution. A broad range of staining 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 2A, lane 3). Wijayanti, Waanders, Bansal and Deeth (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 β -lg and α -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. 2A, lane 4) had a boradly similar electrophoretic pattern to the heated WPI solution without added NaCl (Fig. 2A, lane 3) in the non-reducing

and reducing SDS-PAGE gel. The heated WPI and WPI+NaCl solutions had low levels of monomeric β -lg and α -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. 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. 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 in 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 β -lg and α -lac monomer bands increased considerably for all heated WPI solutions in the reducing gel compared to the intensities 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. 2B), indicating that the larger aggregates (>250 kDa) were formed via disulphide bonding. However, a broad range of staining 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.

3.3.2. 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 small peak being identified (Fig. 3). 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 1). Similar results were obtained by Loveday, Ye, Anema and Singh (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 VMDs of any of the heated solutions, the heated WPI+NaCl solution had slightly larger particles (89 nm) than had 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. 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 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, Rector and Kinsella, 1991). Xiong, Dawson and Wan (1993) reported that the thermal aggregation of β -lg (1.2 mg/ml) occurred at temperatures \geq 76°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, Dawson and Wan (1993) reported that a heated β -lg solution with added NEM and NaCl had the same aggregation profile as the β -Lg solution with added NaCl, highlighting the importance of interactions other than disulphide bonds, e.g. hydrophobic and electrostatic interactions. Brodkorb, Croguennec, Bouhallab and Kehoe (2015) also suggested that blockage of disulphide bonds, by the addition of NEM, may

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

3.3.3. 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. 4A) and no obvious differences were seen 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. 4B), with the later subunits being similar to those in the unheated WPI (Fig. 4A). Mudgal, Daubert, Clare and Foegeding (2010) reported similar results for negatively-stained TEM micrographs of heated β-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., fewer globular subunits visible; Fig. 4C) than the aggregates formed in the WPI solutions heated without added NaCl. Walkenström, Windhab and Hermansson (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 where visible which formed elongated particulate strands. The heated WPI+NEM solution had smaller aggregates (Fig. 4D) than had 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. 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. 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, Mulvihill and Morris (2007) and Verheul, Roefs and de Kruif (1998) reported that, in heated WPI/β-lg solutions, the levels of protein denaturation can be similar while the rate and extent of aggregation may vary, depending on the presence of added NaCl.

3.4. 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, than had the unheated WPI control (Table 1). Chevalier *et al.* (2001), using the OPA method, reported similar results for β -lg solutions (0.4% protein) heated at 60°C for 72 h, which had ~5.0% lower AAG compared to the unheated β -lg solutions. 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 noncovalent interactions (Table 1, Figs. 1 and 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 innate 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. 1B) that requires the nucleophile reactant (e.g., lysine) to be unprotonated (i.e., R-NH₂) (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; there is an increased rate observed with positively-charged adjacent groups, and decreased rate with negatively charged adjacent groups (Fig. 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 (Spellman, McEvoy, O'Cuinn & FitzGerald, 2003). Jacobs, Leburg and Madaj (1986) reported that reactivity of the OPA reagent to amino groups was 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 from that in the unheated WPI solution when measured by the OPA method, indicating that the OPA method is less impacted than is the TNBS method by conformational changes of whey protein molecules on heating.

The heated WPI+NaCl solution was the only solution that had significantly ($P \le 0.05$) 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 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 1, Figs 4D, Fig. 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 more amino groups becoming unavailable 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, Scott & Trepman, 1979).

There was no significant (P > 0.05) difference between the level of AAG in the heated WPI+NEM solutions and that in the unheated WPI control solution when measured by either the OPA or TNBS methods (Table 1). The smaller protein aggregates (Table 1, Figs 2A and 4D) present in the heated WPI+NEM solution, compared to all other heated WPI solutions, were 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, Bansal, Sharma and Deeth (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, than had 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 were 4.3% and 9.0% reductions 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 occured *via* non-covalent bonding, which is promoted by the presence of NaCl on heating of whey protein solutions (Sawyer, 1968; Wijayanti, Waanders, Bansal, & Deeth, 2015).

A comparison of the coefficients 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 was the TNBS method. Morales, Romero and Jiménez-Pérez (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 was the OPA method.

4. Conclusions

The results of this study demonstrate that the OPA method for measuring AAG is less impacted than was the TNBS method by 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, and 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 monitor reactions involving the modification of AAG, including succinylation, methylation, conjugation, hydrolysis, and thiolation of proteins and the findings herein may have implications for those measuring the consumption or liberation of AAG in such studies.

Acknowledgements

The authors would like to acknowledge the Department of Anatomy & Neuroscience Imaging Centre, BioSciences Institute, University College Cork, for assistance in preparing and imaging samples for this research. The authors are grateful to our colleague, Shane Crowley

for the helpful technical discussions on the manuscript. This research was financially supported by the Food Institutional Research Measure (FIRM) initiative of the Irish Department of Agriculture, Food and the Marine (project number 10/RD/OptiHydro/UCC/702). The authors declare no conflict of interest in this study.

References

- Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry*, 27, 1256-1262.
- Anema, S. G. (2009). The whey proteins in milk: Thermal denaturation, physical interactions and effects on the functional properties of milk. In (M. Boland, H. Singh & A. Thompson, Eds.) *Milk Proteins: From Expression to Food*, 2nd Ed. (pp. 269-318)
 Cambridge, MA: Academic Press.
- AOAC. (1995). *Official methods of analysis* (16th ed., Vol. 33, pp. 1-75). Arlington, VA, USA: Association of Official Analytical Chemists.
- Brodkorb, A., Croguennec, T., Bouhallab, S. & Kehoe, J. J. (2015). Heat-induced denaturation, aggregation and gelation of Whey Proteins. In P. L. H. McSweeney and J. A. O'Mahony (Eds.), Advanced Dairy Chemistry. Volume 1B: Proteins: Applied Aspects, 4th Edition (pp. 155-174). New York: Springer.
- Bryant, C. M., & McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science & Technology*, 9, 143-151.

- Cattaneo, S., Masotti, F., & Pellegrino, L. (2009). Liquid infant formulas: technological tools for limiting heat damage. *Journal of Agricultural and Food Chemistry*, 57, 10689-10694.
- Cayot, P., & Tainturier, G. (1997). The quantification of protein amino groups by the trinitrobenzenesulfonic acid method: a reexamination. *Analytical Biochemistry*, 249, 184-200.
- Chen, R. F., Scott, C., & Trepman, E. (1979). Fluorescence properties of *o*-phthaldialdehyde derivatives of amino acids. *Biochimica et Biophysica Acta (BBA)-Protein Structure*, 576, 440-455.
- Chevalier, F., Chobert, J. M., Popineau, Y., Nicolas, M. G., & Haertlé, T. (2001).
 Improvement of functional properties of β-lactoglobulin glycated through the
 Maillard reaction is related to the nature of the sugar. *International Dairy Journal*, 11, 145-152.
- Croguennec, T., Bouhallab, S., Mollé, D., O'Kennedy, B. T., & Mehra, R. (2003). Stable monomeric intermediate with exposed Cys-119 is formed during heat denaturation of β-lactoglobulin. *Biochemical and Biophysical Research Communications, 301*, 465-471.
- Donovan, M., & Mulvihill, D. M. (1987). Thermal denaturation and aggregation of whey proteins. *Irish Journal of Food Science and Technology*, 1, 87-100.
- Fitzsimons, S. M., Mulvihill, D. M., & Morris, E. R. (2007). Denaturation and aggregation processes in thermal gelation of whey proteins resolved by differential scanning calorimetry. *Food Hydrocolloids*, 21, 638-644.

- Finot, P. A. (1983). Chemical modifications of the milk proteins during processing and storage. Nutritional, metabolic and physiological consequences. *Kieler Milchwirtschaftliche Forschungsberichte*, 35, 357–369.
- Giroux, H. J., Houde, J., & Britten, M. (2010). Preparation of nanoparticles from denatured whey protein by pH-cycling treatment. *Food Hydrocolloids*, *24*, 341-346.
- Herwig, N., Stephan, K., Panne, U., Pritzkow, W., & Vogl, J. (2011). Multi-element screening in milk and feed by SF-ICP-MS. *Food Chemistry*, *124*, 1223-1230.
- Hoffmann, M. A., & van Mil, P. J. (1997). Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulfide bonds. *Journal of Agricultural and Food Chemistry*, *45*, 2942-2948.
- Hurrell, R. F., & Carpenter, K. J. (1980). The estimation of available lysine foodstuffs after Maillard reactions. *Progress in Food & Nutrition Science*, *5*, 159-176.
- Ikeda, S., & Morris, V. J. (2002). Fine-stranded and particulate aggregates of heat-denatured whey proteins visualized by atomic force microscopy. *Biomacromolecules*, *3*, 382-389.
- ISO22412. (2008). International Standard, Particle Size Analysis Dynamic Light Scattering, International Organisation for Standardisation (ISO) 2008. Geneva, Switzerland
- Jacobs, W. A., Leburg, M. W., & Madaj, E. J. (1986). Stability of *o*-phthalaldehyde-derived isoindoles. *Analytical Biochemistry*, *156*, 334-340.
- Jiang, Z., & Brodkorb, A. (2012). Structure and antioxidant activity of Maillard reaction products from α-lactalbumin and β-lactoglobulin with ribose in an aqueous model system. *Food Chemistry*, 133, 960-968.

- Joys, T. M., & Kim, H. (1979). *o*-Phthalaldehyde and the fluorogenic detection of peptides. *Analytical Biochemistry*, *94*, 371-377.
- Kitabatake, N., Wada, R., & Fujita, Y. (2001). Reversible conformational change in βlactoglobulin A modified with *N*-ethylmaleimide and resistance to molecular aggregation on heating. *Journal of Agricultural and Food Chemistry*, 49, 4011-4018.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head to bacteriophage T4. *Nature*, 227, 680–685.
- Loveday, S. M., Wang, X. L., Rao, M. A., Anema, S. G., Creamer, L. K., & Singh, H. (2010). Tuning the properties of β-lactoglobulin nanofibrils with pH, NaCl and CaCl2. *International Dairy Journal*, 20, 571-579.
- Majhi, P. R., Ganta, R. R., Vanam, R. P., Seyrek, E., Giger, K., & Dubin, P. L. (2006).
 Electrostatically driven protein aggregation: β-lactoglobulin at low ionic strength.
 Langmuir, 22, 9150-9159.
- Marangoni, A. G., Barbut, S., McGauley, S. E., Marcone, M., & Narine, S. S. (2000). On the structure of particulate gels—the case of salt-induced cold gelation of heat-denatured whey protein isolate. *Food Hydrocolloids*, 14, 61-74.
- Matsudomi, N., Rector, D., & Kinsella, J. E. (1991). Gelation of bovine serum albumin and β-lactoglobulin; effects of pH, salts and thiol reagents. *Food Chemistry*, 40, 55-69.
- Means, G. E., & Feeney, R. E. (1995). Reductive alkylation of proteins. *Analytical Biochemistry*, 224, 1-16.
- Morales, F. J., Romero, C., & Jiménez-Pérez, S. (1995). New methodologies for kinetic study of 5-(hydroxymethyl)-furfural formation and reactive lysine blockage in heat-treated milk and model systems. *Journal of Food Protection*, 58, 310-315.

- Mudgal, P., Daubert, C. R., Clare, D. A., & Foegeding, E. A. (2010). Effect of disulfide interactions and hydrolysis on the thermal aggregation of β-lactoglobulin. *Journal of Agricultural and Food Chemistry*, 59, 1491-1497.
- 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.
- O'Kennedy, B. T., & Mounsey, J. S. (2006). Control of heat-induced aggregation of whey proteins using casein. *Journal of Agricultural and Food Chemistry*, 54, 5637-5642.
- Oldfield, D. J., Singh, H., & Taylor, M. W. (2005). Kinetics of heat-induced whey protein denaturation and aggregation in skim milks with adjusted whey protein concentration. *Journal of Dairy Research*, 72, 369-378.
- Obi, I. U. (1982). Application of the 2, 4, 6-Trinitrobenzene-l-Sulfonic Acid (TNBS) Method for determination of available lysine in maize seed. *Agricultural and Biological Chemistry*, 46, 15-20.
- Rutherfurd, S. M. (2010a). Methodology for determining degree of hydrolysis of proteins in hydrolysates: A review. *Journal of AOAC International*, *93*, 1515-1522.
- Rutherfurd S.M. (2010b). Bioavailability of lysine in heat-treated foods and feedstuffs [PhD thesis]. Wageningen University, Wageningen, the Netherlands. ISBN: 978-90-8585-682-685.

Rutherfurd, S. M., & Moughan, P. J. (2005). Digestible reactive lysine in selected milk-based products. *Journal of Dairy Science*, 88, 40-48.

- Ryan, K. N., Vardhanabhuti, B., Jaramillo, D. P., Van Zanten, J. H., Coupland, J. N., & Foegeding, E. A. (2012). Stability and mechanism of whey protein soluble aggregates thermally treated with salts. *Food Hydrocolloids*, 27, 411-420.
 - Ryan, K. N., Zhong, Q., & Foegeding, E. A. (2013). Use of whey protein soluble aggregates for thermal stability—A hypothesis paper. *Journal of Food Science*, 78, R1105-R1115.
 - Sağlam, D., Venema, P., de Vries, R., & van der Linden, E. (2014). Exceptional heat stability of high protein content dispersions containing whey protein particles. *Food Hydrocolloids*, 34, 68-77.
 - Sawyer, W. H. (1968). Heat denaturation of bovine β-lactoglobulins and relevance of disulfide aggregation. *Journal of Dairy Science*, *51*, 323-329.
 - Skraup, Z. H., & Kaas, K. (1906). Über die Einwirkung von salpetriger Säure auf Ovalbumin. Justus Liebigs. Ann. Chemie, 351, 379-389.
 - Spellman, D., McEvoy, E., O'Cuinn, G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal*, 13, 447-453.
 - Vardhanabhuti, B., Foegeding, E. A., McGuffey, M. K., Daubert, C. R., & Swaisgood, H. E. (2001). Gelation properties of dispersions containing polymerized and native whey protein isolate. *Food Hydrocolloids*, 15, 165-175.
 - Verheul, M., Roefs, S. P., & de Kruif, K. G. (1998). Kinetics of heat-induced aggregation of β-lactoglobulin. *Journal of Agricultural and Food Chemistry*, *46*, 896-903.

- Walkenström, P., Windhab, E., & Hermansson, A. M. (1998). Shear-induced structuring of particulate whey protein gels. *Food Hydrocolloids*, 12, 459-468.
- Wijayanti, H. B., Bansal, N., Sharma, R., & Deeth, H. C. (2014b). Effect of sulphydryl reagents on the heat stability of whey protein isolate. *Food Chemistry*, *163*, 129-135.
- Wijayanti, H. B., Waanders, J., Bansal, N., & Deeth, H. C. (2015). Identification of the binding of β-lactoglobulin (β-Lg) with sulfhydryl (–SH) blocking reagents by polyacrylamide gel electrophoresis (PAGE) and electrospray ionisation/time of flightmass spectrometry (ESI/TOF-MS). *LWT-Food Science and Technology*, 63, 934-938.
- Xiong, Y. L., Dawson, K. A., & Wan, L. (1993). Thermal aggregation of β-lactoglobulin:
 effect of pH, ionic environment, and thiol reagent. *Journal of Dairy Science*, *76*, 70-77.

Fig. 1. Overview of the reaction of (A) *ortho*-phthaldialdehyde (OPA) and (B)

trinitrobenzenesulfonic acid (TNBS) with the amino acid lysine. OPA in the presence of a reducing agent (R'SH) reacts with available amino groups (i.e., terminal amino acids and the ϵ -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.

Fig. 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.

Fig. 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; \blacksquare or \Box) and WPI solutions containing 20 mM added NaCl (WPI+NaCl; Δ), 5 mM *N*-ethylmaleimide (WPI+NEM; \circ) or a mixture of 20 mM added NaCl and 5 mM NEM (WPI+NaCl+NEM; \diamond). Insert shows data for peak 2 with rescaled x- and y-axes.

Fig. 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.

Fig. 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 ortho-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods. The diagram represents unheated, native whey proteins (A), aggregated whey proteins on heating 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.













Table 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 OPA and 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

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

presented as means \pm standard deviation of data from four independent trials.

⁽¹⁾ Means within a column without a common lowercase superscript letter are significantly different ($P \le 0.05$).

⁽²⁾ Means within a row for the same sample without a common uppercase superscript letter are significantly different ($P \le 0.05$).

Highlights

Added NaCl promoted aggregation in heated whey protein solutions N-Ethylmaleimide limited disulfide bond-mediated aggregation an O. S. C. C. B. C. Lower level of available amino groups measured when using TNBS than OPA