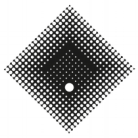

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

Influence of heat and pH on structure and conformation of whey proteins

This is the Accepted version of the following publication

Dissanayake, Muditha, Ramchandran, Lata, Piyadasa, Chathuri and Vasiljevic, Todor (2013) Influence of heat and pH on structure and conformation of whey proteins. International Dairy Journal, 28 (2). pp. 56-61. ISSN 0958-6946

The publisher's official version can be found at
http://www.sciencedirect.com/science/article/pii/S0958694612001987#
Note that access to this version may require subscription.

Downloaded from VU Research Repository https://vuir.vu.edu.au/22190/

Accepted Manuscript

Influence of heat and pH on structure and conformation of whey proteins

Muditha Dissanayake, Lata Ramchandran, Chaturi Piyadasa, Todor Vasiljevic

PII: S0958-6946(12)00198-7

DOI: 10.1016/j.idairyj.2012.08.014

Reference: INDA 3419

To appear in: *International Dairy Journal*

Received Date: 25 March 2012

Revised Date: 6 July 2012

Accepted Date: 21 August 2012

Please cite this article as: Dissanayake, M., Ramchandran, L., Piyadasa, C., Vasiljevic, T., Influence of heat and pH on structure and conformation of whey proteins, *International Dairy Journal* (2012), doi: 10.1016/j.idairyj.2012.08.014.

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.

ACCEPTED MANUSCRIPT

Influence of heat and pH on structure and conformation of whey proteins

Muditha Dissanayake[a], Lata Ramchandran[a], Chaturi Piyadasa[b], Todor Vasiljevic[a,b,*]

[a]*Advanced Food Systems Faculty Research Unit, School of Biomedical and Health Sciences, Faculty of Health, Engineering and Science, Victoria University, Melbourne, VIC, Australia*

[b]*Institute for Sustainability and Innovation, Victoria University, Melbourne, VIC, Australia*

[*]Corresponding author: Tel: +61 3 9919 8062

E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic)

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abstract

The aim of this study was to understand the fundamental interactions responsible for aggregation of whey proteins (WPs) at pH 6 and 3 during heating at 140 ºC for 30 s in the presence of different acidulants. The conformational changes in the various heat-treated WP dispersions were studied using chemical bond blockers and analysed using differential scanning calorimeter thermograms, polyacrylamide gel electrophoresis and turbidity measurements. Overall, the results indicated that WPs were denatured mainly by disruption of hydrophobic interactions, and that the extent of WP denaturation at pH 3 was affected by the type of acidulant used. The type of acidulant affected the extent of formation of additional high or medium molecular weight aggregates during heating at pH 3, while the types of interactions involved in the formation of such aggregates were affected by the pH at heating.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1. Introduction

Whey proteins (WPs) have distinctive nutritional and functional properties that make them unique food ingredients. Different attractive and repulsive molecular forces, involved in the stability of unique three-dimensional structure of proteins (Damodaran, 2008), affect their functionality. These include intrinsic van der Waals and steric forces, as well as electrostatic, hydrogen bonding and hydrophobic interactions that arise from the influence of the surrounding environment. The physico-chemical properties that govern the overall functionality of WPs are a result of intrinsic factors native to the proteins, mainly their structure and conformation, as well as extrinsic factors, such as environmental conditions including pH and temperature. Any processing condition that influences the intrinsic or extrinsic factors will affect protein conformation and thereby influence the functionality of WPs. Therefore, the inclusion of WPs into food systems is dependent on processing conditions applied and their influence on protein structure that, due to the heat sensitivity of WPs, may even result in complete denaturation and thus limit their application. Recently a novel approach to stabilization of WPs through microparticulation was proposed (Dissanayake & Vasiljevic, 2009; Dissanayake, Liyanaarachchi, & Vasiljevic, 2012) to alleviate this problem.

Heating, a common unit operation in food processing, can have an impact on the functionality of WPs since it may induce their denaturation, aggregation and flocculation. Denaturation of WPs results in unfolding of the compact structures, which subsequently causes aggregation mainly due to the exposure of previously buried apolar groups and occurrence of sulfhydryl/disulfide exchange chain reactions via activated thiol groups (Lee, Morr & Ha, 1992). Intrinsic and extrinsic environmental factors, such as protein

ACCEPTED MANUSCRIPT

concentration, pH, temperature, ionic strength and solvent condition, determine the rates and pathways of these physicochemical reactions (Brandenberg, Morr, & Weller, 1992; Iordache & Jelen, 2003; Marangoni, Barbut, McGauley, Marcone, & Narine, 2000), which in turn affects protein functionality. An observed improvement in overrun and stability of foams of WP suspensions at pH 7 when heated to 55 ºC was attributed to denaturation of WP. It was also observed that the availability of proteins to form films and emulsions decreased at higher temperatures, in turn impairing foaming and emulsifying characteristics of the proteins (Phillips, Schulman, & Kinsella, 1990). Denatured dispersions of randomly coiled molecules of proteins have been reported to have greater viscosity than solutions of compact folded globular molecules of the same molecular weight (Damodaran, 2008). Therefore, WPs with similar composition may differ in their functionality depending on their extent of denaturation.

The denaturation step, consisting of successive unimolecular reactions, is usually considered to be a first-order reaction, while the subsequent step of aggregation involves bimolecular and second-order reactions (O'Kennedy & Mounsey, 2009). Aggregation is strongly influenced by attractive and repulsive forces, which are dependent on pH. The free SH group in β-lactoglobulin (β-LG) is activated by conformational changes of the protein between pH 6.5 and 8.0 (Tanford Transition). At neutral pH, the free SH groups are understood to be the dominant mechanism of heat aggregation (Unterhaslberger, Schmitt, Sanchez, Appolonia-Nouzille, & Raemy, 2006), which is not the case under acidic conditions, since free SH groups are thought to be inactive. Also, lowering of pH has been shown to increase the thermal stability of the proteins and it has been suggested that hydrogen bonding is also responsible for such stability (Lucey & Singh, 2003). These stages of protein denaturation, as well as the rates and reaction orders, may be affected by change of pH. Controlling the rate of denaturation, aggregation and colloidalisation may result in WP

ACCEPTED MANUSCRIPT

preparations with improved functionality. For example in a recent study (Dissanayake et al., 2012) a two-order magnitude reduction in particle size of microparticulated whey proteins was reported at low pH, with substantially enhanced solubility and heat stability as compared with that at neutral pH. Therefore, an understanding of fundamental interactions involved during aggregation will help to further modulate processing of functional ingredients so as to incorporate WP as novel ingredients in foods.

This study was designed to understand the fundamental interactions of WPs responsible for protein aggregation at low pH during heating at 140 ºC for 30 s in the presence of different acidulants. These interactions were examined using chemical bond blockers and analysed by a turbidity method, differential scanning calorimetry (DSC) and sodium-dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2. Materials and methods

2.1. Sample preparation

Whey protein retentate (30 % total solids) used in the study was supplied by Warrnambool Cheese and Butter Factory (Warrnambool, Victoria, Australia). The compositional analysis of the retentate was carried out following established AOAC methodology as reported previously (Dissanayake & Vasiljevic, 2009). Three types of WP retentate dispersions were prepared for the experiment: (a) control (no pH adjustment, pH ~ 6); (b) pH adjusted to 3 using citric acid (VWR International, Leicestershire, UK) and (c) pH adjusted to 3 using lactic acid (VWR International, Leicestershire, UK). All samples were adjusted to 7% (w/w) protein by diluting the WP retentate with Milli-Q water. Four chemical bond blockers were used with each of the three types of WP retentate dispersions to assess

ACCEPTED MANUSCRIPT

various molecular interactions: (i) 0.5% polyoxyethylene sorbitan monolauarate (Tween 20) (Sigma, St. Louis, USA) for hydrophobic interactions; (ii) 1% sodium-dodecylsulphate (SDS) (Merck, KGaA, Darmstadt, Germany) for non-covalent interactions; (iii) 20 mM *N*-ethylmaleimide (NEM) (Sigma) and (iv) 10 mM dithiothreitol (DTT) to prevent formation of new covalent bonds. In addition, DTT reduces existing covalent disulfide bonds present in proteins (Havea, Watkinson, & Kuhn-Sherlock, 2009). The samples not containing any of the four chemical bond blockers tested acted as controls. Thus, each of the three WP retentate dispersions prepared had 5 types of sample for analysis.

2.2. Heat treatment of whey protein dispersions

Approximately 3.0 mL samples of WP retentate dispersions were transferred into small glass tubes (10 mm in diameter and 75 mm in length), sealed with rubber stoppers and immersed and shaken in an oil bath (Ratek, Boronia, Victoria, Australia) at 140 °C for 30 s, followed by immediate cooling in an ice bath. The selection of pH and temperature for this study was made on the basis of preliminary studies (data not shown) and recently published data (Dissanayake et al., 2012) that indicated that WP aggregates formed at low pH and high temperature had improved functional properties. After heat treatment, the samples were homogenized by vortexing for 5 s before analysis.

2.3. Polyacrylamide gel electrophoresis

The electrophoretic analysis of heat-treated WP dispersions was performed to fractionate and compare individual proteins present in the samples by native or reducing/non-reducing SDS-PAGE by the method described by Havea, Singh, Creamer, and Campanella

ACCEPTED MANUSCRIPT

(1998), with some minor modifications. All heat-treated WP dispersions and standards were diluted with the treatment buffer (0.125 M Tris-HCl, 4% SDS, 20%, v/v, glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue, pH 6.8). About 6 µL of WP dispersion samples, α-lactalbumin (α-LA), β-LG and 8 µL of molecular weight standards were loaded onto either 4 – 15% Tris-HCl Ready gels (Bio-Rad, Hercules, CA, USA) for native PAGE or 4 – 20% iGels (NuSep, French Forest, NSW, Australia) for SDS PAGE (reducing/non reducing) using a cell (Bio-Rad Protean® II xi) filled with relevant tank buffer. Native PAGE was run at 25 mA while SDS-PAGE gels were run at 50 mA for ~ 50 min. The gels were then placed in de-staining solution I (40% methanol, 7% acetic acid) for 30 min, and stained with staining solution (0.025% Coomassie Brilliant Blue R 250, 40% methanol, 7% acetic acid) for 24 h followed by de-staining in solution I for 1 h and in solution II (5% methanol, 7% acetic acid) until the background became clear. Broad-range pre-stained SDS-PAGE standards (Ref. 161-0318, Bio-Rad) were used to compare the molecular weights. A Fuji Film Intelligent Dark Box II with Fuji Film LAS – 1000 Lite V 1.3 software (Fuji Photo Film Co., Ltd., Tokyo, Japan) was used to obtain gel images and analyse the intensity of protein bands formed.

2.4. Measurement of turbidity

Turbidity of heat-treated WP dispersions was determined to measure the extent of aggregation of the denatured WPs. The heat-treated and cooled samples were immediately diluted to 0.1% (w/w) protein and absorbance measured at 420 nm using a spectrophotometer (Novaspec II, Parmacia LKB, Norfolk, UK). The apparent optical density read at 420 nm was used to express the turbidity of the samples (Ju & Kilara, 1998).

2.5. Differential scanning calorimetry

ACCEPTED MANUSCRIPT

Thermal analysis of WP dispersions was performed using a differential scanning calorimeter (DSC 7, Perkin Elmer, Norwalk, CT, USA) and software (Pyris Manager, v.5.0002) to examine the nature of thermal denaturation of WP, as described by Dissanayake and Vasiljevic (2009). The instrument was calibrated using indium (Tpeak = 155.87 °C, ΔH = 28.234 J g^{-1}) and zinc (T_{peak} = 417.4 °C, ΔH = 93.337 J g^{-1}). About 30 µL of the heat treated WP retentate dispersions was weighed into aluminium pans. An empty pan of equal weight served as the reference and all pans were hermetically sealed before placing in the instrument. The samples were scanned from 25 to 100 °C at a scanning rate of 10 °C min^{-1}, and ΔH values and onset, endset and peak temperatures of the thermograms were recorded.

2.6. Statistical analysis

The data obtained were statistically analysed using a randomised-block full-factorial design with the acidulant at two levels (citric or lactic acid) and bond-blocking chemicals (Tween 20, SDS, NEM and DTT, excluding the control) as the major factors and replicates as blocks. The experimental set was replicated with subsampling and analysed using a general linear model (Dissanayake et al., 2010). The level of significance, *P*, was set at 0.05.

3. Results and discussion

3.1. Thermal analysis of whey protein dispersions

The DSC thermograms (Fig. 1) and parameters that describe the thermal behaviour of WP dispersions under the defined experimental conditions are presented in Table 1. The

ACCEPTED MANUSCRIPT

results (Table 1) showed that the peak/denaturation temperature (T_d) of samples in the acidic medium was significantly ($P < 0.05$) higher than at pH 6, irrespective of the type of chemical bond blocker used, indicating reduced heat denaturation. This observation was in line with the observations of an earlier study that showed enhanced heat stability of WP at low pH (Dissanayake et al., 2012). The relatively low ΔH values of WP dispersion heated at pH 6 further indicated destruction of hydrophobic bonds, resulting in denaturation of WPs (Damodaran, 2008). The lowest ΔH values were for dispersions containing chemical bond blockers, with the exception of SDS when used with lactic acid as acidulant, indicating greater rupturing of hydrophobic interactions and thereby implying greater denaturation of WP under these conditions. The enthalpy of samples with SDS in citric acid and water was comparatively lower than those in the presence of lactic acid, which again confirmed the different thermal behaviour of these samples.

The elevated peak temperatures of WP dispersions in the presence of SDS and lactic acid also indicated improved heat stability of WPs under these conditions. This concurred with the significantly ($P < 0.05$) increased onset and endset temperatures of WP dispersions containing SDS and lactic acid as acidulant. Overall, the reduced onset temperature of WPs in the presence of NEM, DTT and Tween 20 than the corresponding controls indicated facilitated denaturation of WPs, likely mediated by enhanced unfolding via altered intra-molecular hydrophobic, covalent or other interactions.

3.2. Electrophoretic analysis of whey protein dispersions heated in the presence of chemical blockers

Fig. 2 shows the native PAGE patterns of heat-treated WP dispersions at pH 6 and 3 in the presence or absence of the selected chemical bond blockers. Lane 1 for each of the

three types of WP dispersions was the control without any chemical bond blocker addition. The addition of chemical bond blockers affected protein aggregation by preventing formation of different molecular associations or resolving aggregates by breaking the bonds formed. Hydrophobic interactions were affected by Tween 20, while SDS affected all types of non-covalent interactions. NEM prevented formation of new covalent associations due to blockage of thiol groups. In the presence of DTT, all existing intra-molecular disulfide linkages were reduced and formation of new disulfide bonds during heating was prevented, restricting protein aggregation to only non-covalent linkages (Baldwin, 2010; Havea et al., 2009).

Native PAGE patterns of heat-treated WP in the presence of different chemical bond blockers at pH 6 (Fig. 2A) indicated that most of the native WP were denatured, except when they were heated in the presence of SDS. The bands observed at the top of each lane suggested the formation of high molecular weight aggregates, while those labelled (a) and (b) indicated the formation of medium molecular weight aggregates. Since α-LA and β-LG bands are clearly visible only in lane 3 (Fig. 2A), most of the denatured WP apparently aggregated during heating through non-covalent associations, since SDS prevented formation of all types of non-covalent associations. This was further confirmed by the absence of α-LA and β-LG bands in the PAGE gel of WP dispersion containing DTT (Fig. 2A, lane 5). Non-covalent interactions prevail at temperatures above 90 °C and play an important role in the aggregation pathways of proteins (de la Fuente, Singh, & Hemar, 2002). Also, except for the sample containing SDS, the faint protein bands indicating the medium molecular weight aggregates (a) and (b) present in lanes 1, 4 and 5 suggest that such aggregates may have been formed via hydrophobic and non-covalent associations, since they were absent from lanes 2 and 3, i.e., in the WP dispersions containing Tween 20 and SDS. The band corresponding to (b), as well

234 as the two bands present in lane 4 (with NEM), corresponding to even smaller protein
235 aggregates, may be smaller non-covalent WP aggregates.

236 As indicated by the native PAGE pattern of WP dispersion at pH 3 (Fig. 2B,C), the
237 bands representing α-LA and β-LG were visible regardless of the acidulant type, indicating
238 increased stability of WPs against heat-induced denaturation at pH 3 compared with pH 6.
239 WPs are completely denatured when heated at 90 °C for 10 min (O'Connell & Fox, 2003). In
240 addition, some protein aggregation has occurred, as observed by the appearance of bands on
241 top the stacking gels as well those labelled (a) and (b) (Fig. 2B,C). These bands were more
242 pronounced when citric acid (Fig. 2B) was used as the acidulant than in the case of lactic acid
243 (Fig. 2C). This suggested that either WP dispersions that had been adjusted to pH 3 already
244 contained covalently aggregated materials before heating or that formation of other non-
245 covalent associations have been facilitated by existing covalent associations at pH 3. This is
246 contrary to the general understanding that covalent-bond-mediated WP aggregation is very
247 unlikely at pH 3 (Lucey & Singh, 2003). It is generally expected that about 8 – 10%
248 denatured and aggregated proteins may be present in fresh commercial whey protein
249 concentrate products and that some of these aggregates may be formed through covalent
250 interactions (Havea et al., 2009).

251 Non-reducing SDS-PAGE patterns of the heated WP dispersions at pH 6 or 3 in the
252 presence of different chemical bond blocking agents are shown in Fig. 3. As shown in lanes 1
253 and 2 of Fig. 3A, corresponding to the control and the sample with Tween 20, some
254 aggregated materials on top of the stacking gel, as well as resolved protein bands
255 corresponding to α-LA and β-LG, were observed which were absent from the native gels
256 (Fig. 2A). This indicated that WP aggregates formed during heating via both covalent and
257 non-covalent interactions in the control as well as in the presence of Tween 20 where only the
258 hydrophobic interactions were prevented. This was further confirmed by the presence of

ACCEPTED MANUSCRIPT

strong β-LG bands and weaker bands at the top of the stacking gels in lanes 4 and 5 (Fig. 3A), since NEM and DTT prevent formation of additional aggregates via covalent bonds. The sample with SDS, in which non-covalent aggregations were restricted, also confirmed the occurrence of certain degree of covalent aggregation at pH 3 as the intensity of β-LG band was less pronounced (Fig. 3A, lane 3) compared with those in lanes 4 and 5. Furthermore, faint protein bands attributed to medium and low molecular weight aggregates appeared in lanes 1, 2 and 3, but not in lanes 4 and 5, verifying that they were newly formed covalent associations.

Reducing SDS PAGE patterns of heat-treated WPs (Fig. 4) showed that, in the presence of β-mercaptoethanol, WPs formed high molecular weight aggregates when the medium was acidic but not at pH 6 where only protein bands corresponding to α-LA and β-LG appeared. This was more apparent in the presence of lactic acid, as observed by the appearance of intense bands on top of the stacking gel. However, the appearance of two bands corresponding to α-LA may be its two genetic variants that have separated in the acidic pH.

3.3. Turbidity of heated whey protein dispersions

Fig. 5 shows the turbidity of WP dispersions in water (at pH 6) or in the presence of citric or lactic acid (at pH 3) in the presence of Tween 20, SDS, NEM and DTT, and the corresponding control samples after heat-treatment at 140 °C for 30 s. Significant ($P < 0.05$) differences in turbidity were observed when WPs were heated at pH 6. The presence of SDS significantly ($P < 0.05$) lowered the turbidity at pH 6, while the opposite effect was found at pH 3. The lower turbidity observed at pH 6 was due to the ability of SDS to block non-covalent interactions, indicating that WP aggregation occurred mainly *via* non-covalent

ACCEPTED MANUSCRIPT

associations. The extent of aggregate formation was not significantly ($P > 0.05$) influenced by the presence of Tween 20, compared to the control at pH 6. However, the turbidity of WP dispersions containing NEM was equivalent to that of the control, and the turbidity with DTT was significantly higher ($P < 0.05$) than for both the control and sample with NEM. This emphasised that aggregation of WPs under these particular experimental conditions could be driven by other molecular interactions, such as electrostatic, covalent and van der Waals forces. It could be concluded that WP aggregation via non-covalent interactions was facilitated more in the presence of DTT than NEM.

In fact, WP dispersions containing DTT showed highest aggregate formation at pH 6, consistent with the results shown by native PAGE gels (section 3.1). This may be a result of efficient unfolding of WPs, due to cleavage of intra-molecular disulfide covalent linkages by DTT, which in turn facilitated consequent aggregation via non-covalent interactions.

Under acidic conditions (pH 3), the turbidity of all heat-treated WP dispersions was significantly ($P < 0.05$) lower (except WPs with SDS) than at pH 6, implying a significant reduction in aggregation of WPs, which further confirmed the PAGE findings (section 3.1). The type of acidulant, as well as the type of chemical bond blocker used, did not influence the turbidity of the WP dispersions, with the exception of SDS. The increased turbidity observed in the presence of SDS indicated increased aggregation, which could be a consequence of possible complexation of β-LG with SDS at low pH (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008).

4. Conclusions

The current study showed that, during heating of WP dispersions at pH 3 and 6, various interactions were affected, influencing the extent of denaturation of WPs and their

ACCEPTED MANUSCRIPT

consequent aggregation. The results indicated that the denaturation temperatures (T_d) of WPs in acidic medium was significantly ($P < 0.05$) higher than those at pH 6, irrespective of the type of chemical bond blocker used. It was also shown that, during heating at pH 6, most of the WPs denatured and aggregated mainly through non-covalent associations, but that aggregation could be driven by other molecular interactions such as electrostatic, covalent and van der Waals forces. WPs also exhibited increased stability against heat-induced denaturation at pH 3 and formed additional aggregates via covalent interactions before heating or through other non-covalent associations during heating. Reducing-PAGE patterns indicated that WPs had greater tendency to form high molecular weight aggregates when the medium was acidic than at pH 6, which was most apparent when lactic acid was used as acidulant.

Acknowledgements

The authors gratefully acknowledge the financial and technical support from Victoria University. The Warrnambool Cheese and Butter Factory (Warrnambool, Victoria, Australia) is sincerely appreciated for the provision of required raw materials.

References

Baldwin, A. J. (2010). Insolubility of milk powder products – A minireview. *Dairy Science and Technology*, *90*, 169-179.

Brandenberg, A. H., Morr, C. V., & Weller, C. L. (1992). Gelation of commercial whey protein concentrates: effect of removal of low molecular-weight components. *Journal of Food Sci*ence, *57*, 427-432.

ACCEPTED MANUSCRIPT

334 Damodaran, S. (2008). Amino acids, peptides and proteins. In S. Damodaran, K. L. Parkin, &
335 O. R. Fennema (Eds.) *Fennema's food chemistry* (4th edn; pp 217-330). Boca Raton,
336 FL, USA: CRC Press, Taylor and Francis Group.
337 de la Fuente, M. A., Singh, H., & Hemar, Y. (2002). Recent advances in the characterisation
338 of heat-induced aggregates and intermediates of whey proteins. *Trends in Food*
339 *Science and Technology*, *13*, 262-274.
340 Dissanayake, M., & Vasiljevic, T. (2009). Functional properties of whey proteins affected by
341 heat treatment and hydrodynamic high-pressure shearing. *Journal of Dairy Science*,
342 *92*, 1387-1397.
343 Dissanayake, M., Liyanaarachchi, S., & Vasiljevic, T. (2012). Functional properties of whey
344 proteins microparticulated at low pH. *Journal of Dairy Science, 95*, 1667-1679.
345 Havea, P., Singh, H, Creamer, L. K., & Campanella, O. H. (1998). Electrophoretic
346 characterization of the protein products formed during heat treatment of whey protein
347 concentrate solutions. *Journal of Dairy Research, 65*, 79-91.
348 Havea, P., Watkinson, P., & B. Kuhn-Sherlock. (2009). Heat induced whey proteins gels:
349 Protein–protein interactions and functional properties. *Journal of Agricultural and*
350 *Food Chemistry*, *57*, 1506-1512.
351 Iordache, M., & Jelen, P. (2003). High Pressure microfluidization treatment of heat denatured
352 whey protein for improved functionality. *Innovative Food Science and Emerging*
353 *Technologies, 4*, 367-376.
354 Ju, Z. Y., & Kilara, A. (1998). Aggregation induced by calcium chloride and subsequent
355 thermal gelation of whey protein isolate. *Journal of Dairy Science*, *81*, 925-931.
356 Jung, J., Savin, G., Pouzot, M., Schmitt, C., & Mezzenga, R. (2008). Structure of heat-
357 induced β-lactoglobulin aggregates and their complexes with sodium-dodecyl sulfate.
358 *Biomacromolecules, 9*, 2477-2486.

ACCEPTED MANUSCRIPT

359 Lee, S., Morr, C. V., & Ha, E. Y. W. (1992). Structural and functional properties of caseinate
360 and whey protein isolate as affected by temperature and pH. *Journal of Food Science*,
361 *57*, 1210-1214.
362 Lucey, J. A., & Singh, H. (2003). Acid coagulation of milk. In P. F. Fox, & P. L. H.
363 McSweeney (Eds.) *Advanced dairy chemistry – Proteins* (Vol. 1, 3rd edn., Part B; pp
364 1001-1026). New York, NY, USA: Kluwer Academic/Plenum Publishers.
365 Marangoni, A. G., Barbut, S., McGauley, S. E., Marcone, M., & Narine S. S. (2000). On the
366 structure of particulate gels-the case of salt-induced cold gelation of heat-denatured
367 whey protein isolate. *Food Hydrocolloids*, *14*, 61-74.
368 O'Connell, J. E., & Fox, P. F. (2003). Thermal denaturation, aggregation and gelation of
369 whey proteins. In P. F. Fox, & P. L. H. McSweeney (Eds.) *Advanced dairy chemistry*
370 *– Proteins* (Vol. 1, 3rd edn., Part B; pp 1261-1288). New York, NY, USA: Kluwer
371 Academic/Plenum Publishers.
372 O'Kennedy, B. T., & Mounsey, J. S. (2009). The dominating effect of ionic strength on the
373 heat-induced denaturation and aggregation of β-lactoglobulin in simulated milk
374 ultrafiltrate. *International Dairy Journal*, *19*, 123–128.
375 Phillips, L. G., Schulman, W., & Kinsella, J. E. (1990). pH and heat treatment effects on
376 foaming of whey protein isolate. *Journal of Food Science, 55*, 1116-1119.
377 Unterhaslberger, G., Schmitt, C., Sanchez, C., Appolonia-Nouzille, C., & Raemy, A. (2006).
378 Heat denaturation and aggregation of β-lactoglobulin enriched WPI in the presence of
379 arginine HCl, NaCl and guanidinium HCl at pH 4.0 and 7.0. *Food Hydrocolloids*, *20*,
380 1006-1019.

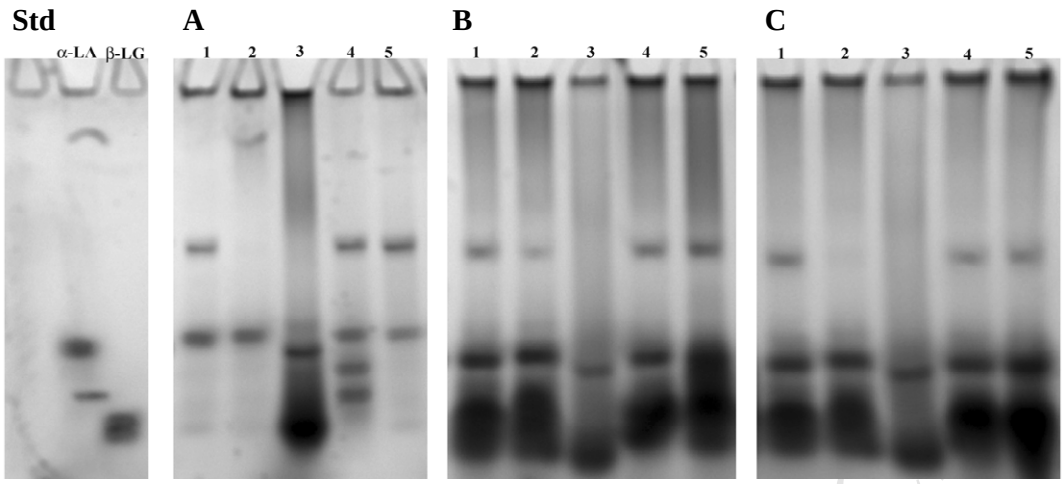
ACCEPTED MANUSCRIPT

Table 1.

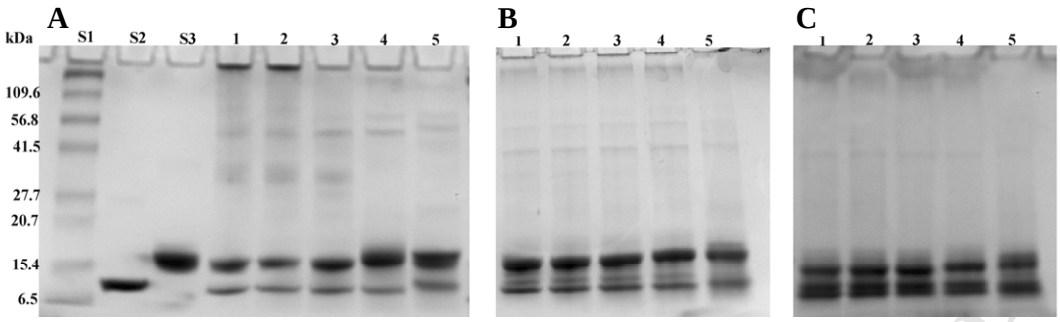
Thermal analysis of whey protein dispersions containing 7% protein at pH 6 (prepared in water) or pH 3 (pH adjusted with citric or lactic acid) in the presence or absence of chemical blockers. [a]

Medium	Chemical	Temperature (°C)		T_d (°C)	ΔH (J g^{-1})
		Onset	Endset		
Water (pH ~ 6)	Control	66.2^{b}	81.4^{a}	74.2^{a}	3.3^{bcd}
	Tween 20	60.7^{a}	82.8^{a}	76.8^{a}	3.6^{bcde}
	SDS	64.4^{b}	83.3^{a}	80.7^{b}	0.6^{a}
	NEM	64.5^{b}	80.9^{a}	73.3^{a}	2.3^{abc}
	DTT	65.9^{b}	81.2^{a}	74.5^{a}	3.3^{bcd}
Citric acid (pH 3)	Control	72.9^{cd}	91.2^{bc}	81.5^{b}	4.3^{cde}
	Tween 20	72.1^{c}	92.7^{bc}	83.2^{b}	6.0^{ef}
	SDS	73.4^{cd}	88.4^{b}	83.5^{b}	1.2^{ab}
	NEM	66.8^{b}	90.7^{bc}	80.3^{b}	7.1^{a}
	DTT	67.3^{b}	96.6^{c}	82.9^{b}	10.0^{g}
Lactic acid (pH 3)	Control	69.8^{bc}	94.8^{c}	81.4^{b}	4.7^{cdef}
	Tween 20	69.4^{bc}	90.4^{bc}	82.1^{b}	5.2^{def}
	SDS	77.7^{d}	111.7^{d}	95.8^{c}	14.5^{h}
	NEM	65.6^{b}	86.7^{b}	78.8^{b}	5.1^{def}
	DTT	71.5^{c}	88.6^{b}	80.3^{b}	5.1^{def}

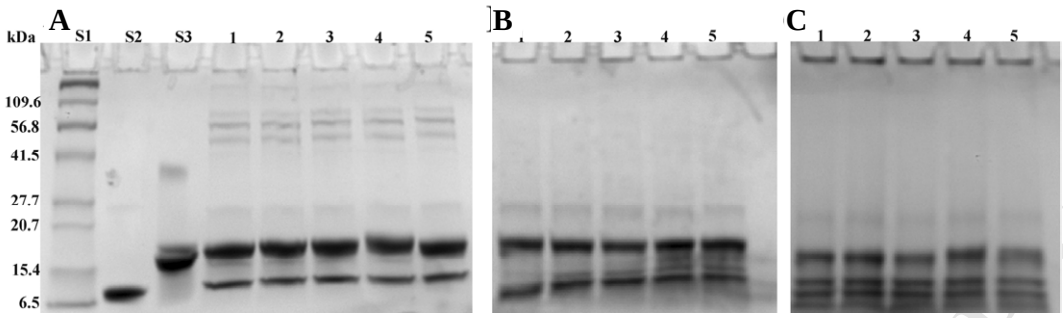
[a] Abbreviations: T_d, denaturation temperature of whey proteins; ΔH, denaturation enthalpy. Values are the average of at least 4 independent observations (n≥4); values with different superscript letters in a column were significantly different ($P < 0.05$). Pooled standard errors of the mean are 1.71, 1.82, 1.40 and 1.26 for onset temperature, endset temperature, T_d and ΔH, respectively.



ACCEPTED MANUSCRIPT

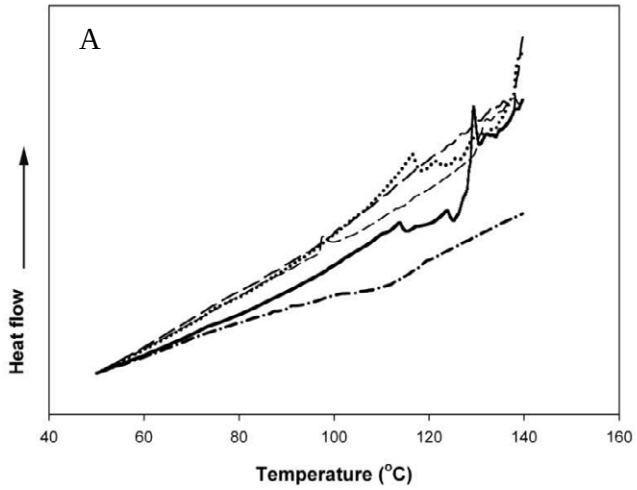


ACCEPTED MANUSCRIPT

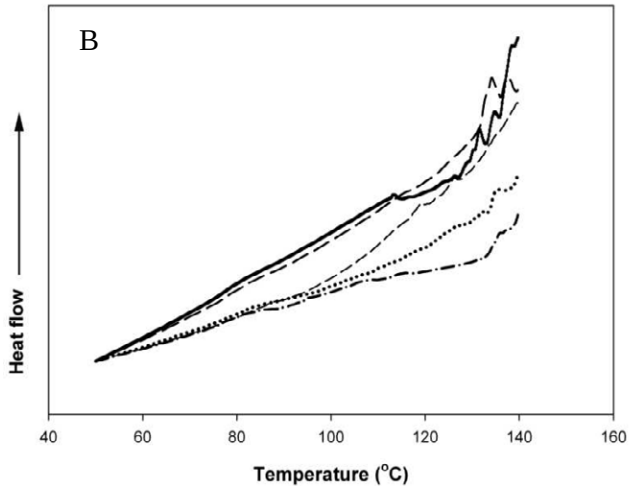


ACCEPTED MANUSCRIPT

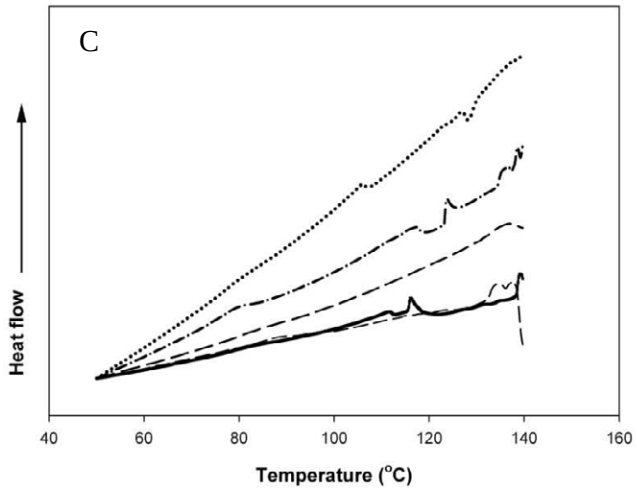
1 Figure 1.



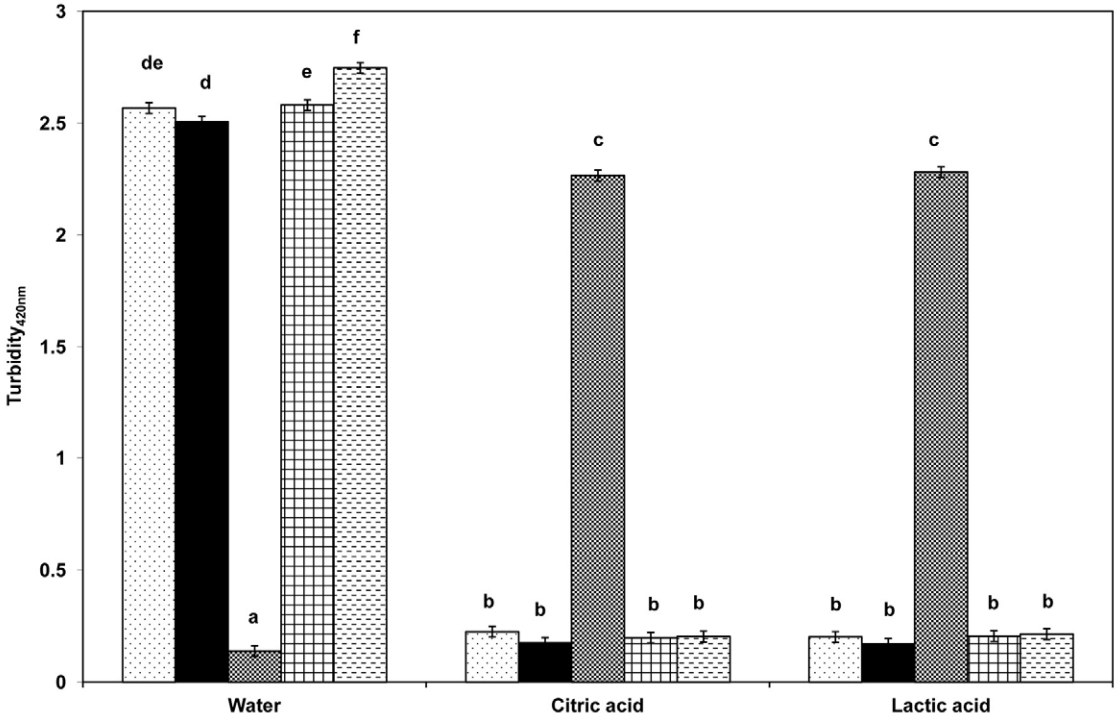
2



3



4



ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure legends

Fig. 1. DSC thermograms of whey protein dispersions at pH 6 with water (A; control) and adjusted to pH 3 using citric acid (B) or lactic acid (C) in the presence of different chemical bond blockers: control (——); Tween20 (— —); SDS (- - -); NEM (· – · –); DTT (·····).

Fig. 2. Non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis patterns of whey protein (WP) dispersions (7%, w/w, protein) in water (panel A; pH not adjusted) and at pH 3 in citric acid solution (panel B) or lactic acid solution (panel C) after heating at 140 °C for 30 s. The bands given by α-lactalbumin and β-lactoglobulin are shown in a separate panel (Std). For panels A, B and C: lane 1, WP dispersion with no chemical bond blocker; lanes 2, 3, 4 and 5, WP dispersions with Tween 20, SDS, NEM and DTT, respectively. Bands labelled (a) and (b) indicated the formation of medium molecular weight aggregates.

Fig. 3. Reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis patterns of whey protein (WP) dispersions (7%, w/w, protein) in water (panel A; pH not adjusted) and at pH 3 in citric acid solution (panel B) or lactic acid solution (panel C) after heating at 140 °C for 30 s. Lane S1, molecular mass markers; lane S2, α-lactalbumin; lane S3, β-lactoglobulin; lane 1, WP dispersion with no chemical bond blocker; lanes 2, 3, 4 and 5, WP dispersions with Tween 20, SDS, NEM and DTT, respectively.

Fig. 4. Native polyacrylamide gel electrophoresis patterns of whey protein (WP) dispersions (7%, w/w, protein) in water (panel A; pH not adjusted) and at pH 3 in citric acid solution (panel B) or lactic acid solution (panel C) after heating at 140 °C for 30 s. Lane S1, molecular mass markers; lane S2, α-lactalbumin; lane S3, β-lactoglobulin; lane 1, WP dispersion with no chemical bond blocker; lanes 2, 3, 4 and 5, WP dispersions with Tween 20, SDS, NEM and DTT, respectively.

Fig. 5. Heat-induced changes in turbidity of 7% (w/w) whey protein dispersions after heat treatment at 140 °C for 30 s at pH 6 (in water) or at pH 3 (in the presence of citric acid or lactic acid) without a chemical bond blocker (control, ⊡), or in the presence of Tween 20 (■), SDS (▩), NEM (▦) and DTT (⊟). Reported data are the means of at least 4 independent observations (n≥4); error bars represent standard error of means.

ACCEPTED MANUSCRIPT

References

Baldwin, A. J. (2010). Insolubility of milk powder products – A mini review. *Dairy Science and Technology*, 90, 169 – 179..

Brandenberg, A. H., Morr., C. V. & Weller, C. L. (1992). Gelation of commercial whey protein concentrates: effect of removal of low molecular-weight components. *Journal of Food Sci*ence, 57, 427-432.

Damodaran, S. (2008). Amino acids, peptides and proteins. In Damodaran, S. Parkin, K. L. & Fennema, O. R. (Eds.), *Fennema's Food Chemistry* (4th ed.) (pp 217-330), CRC Press, Taylor and Francis Gp, Boca Raton, FL, USA.

de la Fuente, M. A., Singh, H. & Hemar, Y. (2002). Recent advances in the characterisation of heat-induced aggregates and intermediates of whey proteins. *Trends in Food Science and Technology*, 13, 262- 274.

Dissanayake, M. & Vasiljevic, T. (2009). Functional properties of whey proteins affected by heat treatment and hydrodynamic high-pressure shearing. *Journal of Dairy Science*, 92, 1387-1397.

Dissanayake, M., Kelly, A. L., & T. Vasiljevic (2010). Gelling properties of microparticulated whey proteins. *Journal of Agriculture and Food Chemistry*, 58, 6825–6832

Dissanayake, M., Liyanaarachchi, S. & Vasiljevic, T. (2012). Functional properties of whey proteins microparticulated at low pH. *Journal of Dairy Science*, 95, 1667-1679.

Havea, P., Singh, H, Creamer, L. K., & Campanella, O. H. (1998). Electrophoretic characterization of the protein products formed during heat treatment of whey protein concentrate solutions. *Journal of Dairy Research, 65*, 79-91.

Havea, P., Watkinson, P. & B. Kuhn-Sherlock. (2009). Heat induced whey proteins gels: Protein–protein interactions and functional properties. *Journal of Agricultural and Food Chemistry*, 57, 1506-1512.

Iordache, M. & Jelen, P. (2003). High Pressure microfluidization treatment of heat denatured whey protein for improved functionality. *Innovative Food Science and Emerging Technologies*, 4, 367-376.

ACCEPTED MANUSCRIPT

Ju, Z. Y. & Kilara, A. (1998). Aggregation induced by calcium chloride and subsequent thermal gelation of whey protein isolate. *Journal of Dairy Science*, 81, 925-931.

Jung, J., Savin, G., Pouzot, M., Schmitt, C. & Mezzenga, R. (2008). Structure of heat-induced β-lactoglobulin aggregates and their complexes with sodium-dodecyl sulfate. *Biomacromolecules*, 9, 2477-2486.

Lee, S., Morr, C. V. & Ha, E. Y. W. (1992). Structural and functional properties of caseinate and whey protein isolate as affected by temperature and pH. *Journal of Food Science*, 57, 1210-1214.

Lucey, J. A. & Singh, H. (2003). Acid coagulation of milk. In Fox, P. F. & McSweeney, P. L. H. (Eds.) *Advanced Dairy Chemistry – Proteins*, Vol. 1, 3rd edition, Part B (pp 1001-1026), Kluwer Academic/Plenum Publishers, New York, USA.

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.

O'Connell, J. E. & Fox, P. F. (2003). Thermal denaturation, aggregation and gelation of whey proteins. In Fox, P. F. & McSweeney, P. L. H. (Eds.) *Advanced Dairy Chemistry – Proteins*, Vol. 1, 3rd edition, Part B (pp 1261-1288), Kluwer Academic/Plenum Publishers, New York, USA.

O'Kennedy, B. T. & Mounsey, J. S. (2009). The dominating effect of ionic strength on the heat-induced denaturation and aggregation of â-lactoglobulin in simulated milk ultrafiltrate. *International Dairy Journal*, 19, 123–128.

Phillips, L. G., Schulman, W. & Kinsella, J. E. (1990). pH and Heat Treatment Effects on Foaming of Whey Protein Isolate. *Journal of Food Science*, 55, 1116-1119.

Unterhaslberger, G., Schmitt, C., Sanchez, C., Appolonia-Nouzille, C. & Raemy, A. (2006). Heat denaturation and aggregation of â-lactoglobulin enriched WPI in the presence of arginine HCl, NaCl and guanidinium HCl at pH 4.0 and 7.0. *Food Hydrocolloids*, 20, 1006-1019.