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ABSTRACT

The effects of heat induced denaturation and subsequent aggregation of Whey Protein Isolate (WPI) solutions on the rate of enzymatic hydrolysis was investigated. Denaturation of whey proteins was monitored by reversed-phase and size exclusion HPLC and observed by native- and SDS-PAGE. Treated and un-treated WPI solutions (100 g L^{-1} protein) were hydrolysed to a target degree of hydrolysis (DH) of 5 % with Corolase® PP. Aggregate formation was monitored using light microscopy, with size distribution determined by particle size. Viscosity and surface hydrophobicity exhibited large increases with heat-treatment and the major protein components in WPI showed differences in their rates of aggregation. Results revealed an increased rate of hydrolysis of protein solutions, which were subjected to a pre-hydrolysis heat-treatment. Light and Confocal Laser Scanning Microscopy (CLSM) images illustrated the optical clarification of the solution, weakening of the gel network and disintegration of aggregates indicative of hydrolysis. Comparison of samples where there was a heat-treatment prior to hydrolysis and a control non-treated hydrolysis reaction, revealed significant differences in the time to reach 5 %DH ($P < 0.001$). The heat-treatments ≥ 75 ºC for 5 min produced significantly ($P < 0.001$) more rapid reactions than the other 5 heat-treatments and the control un-treated reaction. The viscosity, surface hydrophobicity, and insolubility of the heat-treated WPI solutions subsequently declined upon their hydrolysis. The extensive aggregation in some heat-treated solutions was postulated to relate to the congruent increased rate of hydrolysis. This study demonstrated that prior thermal treatment of ≥ 75 ºC for 5 min can accelerate the enzymatic hydrolysis reaction of WPI with Corolase® PP.

INTRODUCTION

Denaturation of globular proteins can provide an altered substrate for subsequent enzymatic hydrolysis. Detailed information on the contribution of thermal denaturation to substrate structure allows for a better understanding of the events taking place during the hydrolysis process.

The conformation adopted by a protein under a particular set of environmental conditions is a delicate balance between forces that promote and hinder un-folding, e.g., hydrophobic interactions (*1*). The physicochemical factors which affect protein aggregation are well documented. Globular proteins retain native conformation within a particular temperature range. Whey proteins, in particular, are sensitive to unfolding at temperatures above 60 ºC (*2*). Whey protein conformational changes arising from heat-treatment may quickly progress to a point where aggregation takes place. The increased thermal motion of heated whey proteins in solution results in disruption of various intra- and intermolecular bonds and exposure of previously 'buried' hydrophobic residues to solvent (*3, 4*). Whey proteins, which have a large proportion of hydrophobic residues, conform structurally to a low surface area-to-volume ratio in order to minimise exposure of hydrophobic (apolar) residues to solvent.

Extended exposure to temperatures > 60 ºC can irreversibly affect the solubility of whey proteins (*2*) and change the relative hydrophobicity at the protein surface leading to exposure of non-polar hydrophobic residues thereby increasing hydrophobic attraction. Hydrophobic interactions are reported to increase with increasing temperature up to ~ 70 ºC, after which they diminish (*1*). Exposure of sulphydryl groups and hydrophobic patches due to unfolding decreases protein stability and as a result solubility (*5, 6*).

The potential for interaction among the individual whey protein fractions during thermal treatments also needs to be taken into consideration. The individual fractions that constitute the whey proteins differ in their thermal stability in the order Ig < BSA < β-lg < α-la < PP (*7*). While heating native α-la on its own does not produce aggregates at temperatures ≤ 75 ºC, free cysteine residues in β-lg and BSA lead to di-sulphide interchange reactions with other β-lg / BSA molecules and with α-la (*8*). Unfolded proteins may be more susceptible to protein-protein interactions via calcium bridging in addition to hydrophobic bonding as well as disulphide interchange reactions (*9*).

The conformational state of a protein is known to affect its rate of proteolysis (*10, 11*). Native globular proteins assume a conformational state which may render them somewhat resistant to proteolysis. Heat-treatment of whey protein substrates has been shown to increase the rate of, or induce, proteolysis (*12-14*), as a result of protein unfolding and di-sulphide bond reduction (*15*). For example, native β-lg is resistant to hydrolysis by pepsin, however, heat-treatment of β-lg at 82 ºC results in peptic hydrolysis (*12*). The exposure of hydrophobic residues can lead to greater reactivity (*4*) and heat-denatured whey proteins can be readily solubilised during hydrolysis (*16*).

It is noteworthy that not all thermal-treatments result in an increase in hydrolytic susceptibility (*17, 18*). Pre-heating of α-la at 95 ºC for 10 min resulted in reduced hydrolysis activity with Pronase®, trypsin and pancreatin activities (*17*). This highlights the inter-dependence of pre-treatment and of substrate conformation with the rate of hydrolysis.

While the choice of protease and the degree of hydrolysis are major determining factors in hydrolysate physico-chemical properties, the function of pre-

hydrolysis heat-treatments in altering these parameters requires clarification. The objective of this study was to investigate the role of thermal aggregation on the hydrolysis of Whey Protein Isolate (WPI) with Corolase® PP a food-grade porcine pancreatic proteolytic preparation. A novel approach employed herein was the use of macrostructure imaging techniques to characterise changes in protein aggregate structures during the course of enzymatic hydrolysis.

MATERIALS AND METHODS

Materials

Whey Protein Isolate (Isolac®) was provided by Carbery Food Ingredients, (Ballineen, Co. Cork, Ireland). The powder contained 89.3 % (w/w) protein by Kjeldahl [*N* x 6.38, (*19*)] comprising 56.5 % β-lactoglobulin, 14.3 % α-lactalbumin, 10.3 % glycosylated caseinomacropeptide (CMP), 8.0 % non-glycosylated CMP, 1.3 % lactoferrin (LF) and 1.7 % bovine serum albumin (BSA). Denatured material amounted to 15.4 % of total protein as determined by urea-denaturing RP-HPLC (*20*).

The digestive-enzyme complex Corolase® PP (E.C. 3.4.21.4.) was from AB Enzymes GmbH (Darmstadt, Germany), and has a minimum activity of 220,000 Lohlein-Volhard Units g^{-1} at pH 8. Corolase® PP possesses chymotrypsin, elastase and tryptic activities as well as aminopeptidase, along with carboxypeptidase A1, A2 and B exopeptidase activities (*21*). Corolase® PP also contains dipeptidase activity and the optimum pH for overall activity is 8.0 (AB Enzymes).

Dialysis membranes (molecular wt. cut off 3500Da) and acetonitrile were purchased from ThermoFisher Scientific (Waltham, MA, USA). All further chemicals were analytical grade and were purchased from Sigma-Aldrich (Dublin, Ireland).

Heat treatment of WPI solutions

WPI solutions (20 mL, 100 g L^{-1} protein, pH 6.4) were subjected to selected heat-treatments over a range of temperatures from 60 °C to 80 °C for selected time periods i.e., 5, 10 and 15 min. The specific temperature (ºC) x time (min) treatments were as follows; 60 x 15, 65 x 5, 65 x 15, 70 x 5, 70 x 15, 75 x 5, 75 x 15, and 80 x 10. Samples were solubilised in de-ionised water and allowed to hydrate overnight at 4 ºC. Heat-treatments were performed in triplicate in a thermostatically controlled water bath. At the end of each treatment solutions were placed in ice/water bath. Lyophilised powders were stored in a cool low moisture environment.

Chromatographic characterisation of control and heated-treated WPI solutions

High performance liquid chromatography (HPLC) was carried out using a Waters 2695 separation module, a Waters 2487 dual wavelength absorbance detector running on Waters Empower® software (Milford, MA, USA). Reversed-phase (RP) HPLC was used to observe the loss in native protein using a Source™ 5RPC, 150 mm x 4.6 mm, column (GE Healthcare, Buckinghamshire, UK). Solvent A was 0.1% trifluoroacetic acid (TFA) in MilliQ water and Solvent B was 90% HPLC-grade acetonitrile (MeCN) containing 0.1% TFA in MilliQ water. Gradient elution conditions were as follows; Solvent B: 20% to 40% in 10 min, 40% to 60% in 20 min, 60 to 100% in 5 min, 100% for 3 min, 100 to 20 % in 3 min, and 20 % for 5 min at a flow-rate of 0.8 mL min^{-1}. Protein solutions (20 μL, 2.5 g L^{-1}) were loaded onto the column which was equilibrated at 28 °C. The column eluate was monitored at 214 nm. Proteins which possess the same retention time as procured non-heat treated standards under gradient elution were designated ‘native’. These whey protein standards were α-la, β-lg A and B, BSA, lactoferrin, and CMP (Sigma-Aldrich,

Dublin, Ireland) and all possessed less than 4 % denatured material by urea-denaturing RP-HPLC (*20*). According to the methodology of Beyer and Kessler (1989), only native proteins would possess the identical retention time as these standards (*22, 23*). Changes in tertiary structure leading to a shift in retention time led to peaks being described as 'non-natively conformed'.

Not all 'non-native' proteins are involved in aggregation and size-exclusion chromatography (SEC) allowed for molecules with a molecular weight (M_w) greater than that of the native protein to be designated as 'aggregates'. SEC was carried out on a TSK Gel G2000SW_{XL}, 7.8 mm x 300 mm, column (TosoHaas Bioscience GmbH, Stuttgart, Germany) using an isocratic gradient of 20 mM sodium phosphate buffer at pH 7 at a flow-rate of 0.5 mL min^{-1} over 60 min. Ribonuclease A, Cytochrome C, Aprotinin, Bacitracin, His-Pro-Arg-Trp, Leu-Trp-Met-Arg, Bradykinin, Leu-Phe, and Tyr-Glu (Bachem AG, Bubendorf, Switzerland) were used as M_w standards along with samples of the previously described whey protein standards. All chromatography test samples and standards were made up in MilliQ water (2.5 g L^{-1} solutions) pre-filtered through 0.45 μm low protein binding membrane filters (Sartorius Stedim Biotech GmbH, Germany) and 20 μL applied to the column. The column elute was monitored at 214 nm and all solvents were filtered under vacuum through 0.45 μm high velocity filters (Millipore (UK) Ltd., Durham, UK).

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a modification of the method of Laemmli (*24*). SDS-PAGE of the heat-denatured WPI samples was carried out under reducing and non-reducing

conditions. The acrylamide for the resolving gel (15 % w/v) was prepared in 1.5 M Tris-HCl buffer, pH 8.8, with the stacking gel (4 % w/v) prepared in 0.5 M Tris-HCl buffer, pH 6.8. Samples were diluted to 3 g L^{-1} in 0.5 M Tris-HCl buffer. The addition of 2-mercaptoethanol for reducing samples was followed by heating at 95 ºC for 5 min.

Native-PAGE gels were prepared using a modification of the method of Manderson et al. (*25*). Before pouring the gels, 50 μL of ammonium persulphate solution (10 % w/v) and 5 μL of *N,N,N',N'*-tetramethylethylenediamine (TEMED) were added to both the separating and stacking gel solutions. Test samples were diluted to 3 g L^{-1} in 0.5 M Tris-HCl buffer.

For both SDS- and native-PAGE the samples were diluted (1:8 v/v) in the respective sample buffers and run in a mini Protean II electrophoresis system (Bio-Rad Alpha Technologies, Dublin, Ireland). For SDS-PAGE the running conditions were 155 V for 50 min and for native-PAGE the conditions were 180 V for 105 min. Staining was with Coomassie Brilliant Blue R-250 in an acetic acid:isopropanol:H_2O solution (3:10:17 by vol.) and gels were destained in an acetic acid:isopropanol:H_2O solution (3:10:17 by vol.). Molecular weights were determined by comparison to a M_w standard (Bio-Rad SDS-PAGE Standards – Low range, Bio-Rad, CA, USA). Imaging was accomplished with a Kodak Image Station 440 CF (Carestream Molecular Imaging, Woodbridge, CT, USA) with accompanying software.

Particle size and surface hydrophobicity

Particle size analysis was carried out using a Malvern® Mastersizer MSS (Malvern Instruments Ltd., Worcestershire, UK) running on Malvern® software. The Mastersizer was fitted with a He-Ne laser measuring at 633 nm and samples were

dispersed in the Malvern® Hydro SM small volume sample dispersion unit (225 x 80 x 180 mm) with a maximum sample capacity of 120 mL. The solutions were diluted in de-ionised water to give a laser obscuration of between 14 and 19 % for optimal detection. Each sample was analysed in triplicate and the D. v 09 was presented herein. The D. v 09 is the representative diameter where 90 % of the measured particles possess a diameter less than or equal to the stated value.

Surface hydrophobicity was determined using a modification of the method of Kato et al. (*26*). Samples of WPI were diluted in sodium dihydrogen phosphate dihydrate buffer (0.02 M, pH 6), with SDS (0.0404 g L^{-1}), and methylene blue (0.0240 g L^{-1}) solutions also prepared in the sodium phosphate buffer. WPI samples (1 g L^{-1}) were mixed (1:2 v/v) with SDS solution and dialysed (MWCO 3.5kDa) against sodium dihydrogen phosphate dehydrate buffer (1:25) for 24 h at 20 ºC. Mixtures of 0.5 mL of dialysate, 2.5 mL of methylene blue and 10 mL of chloroform were centrifuged at 2,500 x g for 5 min. The extinction of the chloroform phase was assessed at a wavelength of 655 nm according to the method of Hiller & Lorenzen (*27*). Chloroform served as the solvent blank and a calibration curve was created for 0 – 100 μg SDS. Surface hydrophobicity (SH) was then determined according to Eqn. 1. Analysis was carried out in duplicate.

$$SH\ (\mu g\ SDS/500\ \mu g\ protein) = (Extinction\ (\lambda = 655\ nm) - 0.0392) / 0.0178 \quad \text{(Eqn. 1)}$$

Where: 0.0392 and 0.0178 are derived from the calibration curve of SDS

Solubility and turbidity

For analysis of turbidity, samples (500 μL) were diluted in 20 mL de-ionised water and the turbidity of the control, heat-treated and hydrolysate samples was determined by the optical density at 550 nm (OD_{550}) using a Varian Cary® 1 dual beam UV-visible spectrophotometer (Varian Ltd., Walton-on-Thames, UK). Optical density was measured at 550 nm so as to reduce the influence of the proteins on the absorbance of the incident light. For turbidity two aliquots of each replicate was analysed.

The solubility of WPI (100 g L^{-1} protein, pH 6.4) solutions subjected to the heat-treatments outlined earlier and the subsequent hydrolysates was determined. Hydrolysates were adjusted to pH 6.4 with 1 N HCl. All test samples were centrifuged at 1330 x g for 30 min at 20 ºC in an Eppendorf 5810 R centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was removed and filtered through Whatman no. 1 (Whatman International Ltd., Kent, UK) cellulose filter paper, after which protein concentration was determined by Kjeldahl (*N* x 6.38). Solubility was expressed as the amount of protein present in the supernatant relative to the total protein of the WPI prior to heat treatment (% w/w). Solubility experiments were performed in duplicate.

Enzymatic hydrolysis

Degree of hydrolysis (DH) is the number of peptide bonds cleaved (*h*) as a percentage of total peptide bonds (h_{tot}) and can be related to the consumption of base as hydrolysis releases protons by the following formula (*28*):

$$DH\% = 100 \cdot B \cdot N_B \cdot (1/\alpha) \cdot (1/MP) \cdot (1/h_{tot}) \quad \text{(Eqn. 2)}$$

Where: B is the volume of base, N_B is the normality of the base, $1/\alpha$ is the average degree of dissociation of α-NH_2 residues at pH 8 and 50 ºC, MP is the mass of protein (g) and h_{tot} is the total number of peptide bonds given in meq g^{-1} (N x f_N). The h_{tot} for whey protein concentrates is 8.8.

For all hydrolysis experiments, 300 mL solutions of WPI (100 g L^{-1} protein, pH 8) were hydrolysed to a target degree of hydrolysis (DH) of 5 % with Corolase® PP unless stated otherwise. Hydrolysis was performed at an enzyme:substrate (E:S) ratio of 1:100 (w/w) on a protein equivalent basis and the hydrolysis conditions were 50 ºC and pH 8, controlled throughout the reaction. The reaction was initiated by the addition of 10 mL of Corolase® PP solution containing 0.3 g Corolase® PP giving a final E:S of 1% (w/w). The pH was controlled by titration with 2 N NaOH using a Metrohm 842 Titrando dosing unit (Metrohm Ltd., Herisau, Switzerland) and the reaction was agitated utilising an over-head stirrer at 300 rpm. The reaction was terminated by heating the enzyme at 85 ºC for 20 min. All hydrolysis experiments were conducted in triplicate.

For hydrolysis experiments performed at enzyme:substrate ratios of 0.5:100 (0.5% w/w), 1%, 1.5%, 1.75%, 2%, 2.5%, and 3% (w/w) the hydrolysis conditions were the same as previously described.

Rheological analysis

Rheological analysis of heat-denatured WPI and hydrolysates of control and heat-denatured WPI was carried out using an AR G2 rheometer (TA Instruments, Crawley, UK), equipped with a starch pasting cell (cell diameter 36.00 mm) complete with impeller, rotor diameter 32.40 mm, rotor length 12.00 mm (TA Instruments,

Crawley, UK). Samples (28 g, 100 g L^{-1} protein) were first pre-sheared at 16.57 rad s^{-1} for 1 min and equilibrated for 1 min at 25 ºC. Samples were then sheared at a constant value of 16.57 rad s^{-1} and the viscosity measured after exactly 5 min of shearing. Rheological measurements of heat-denaturation of WPI were taken at intervals between 25 and 80 °C. Un-heated control and heat-treated samples which were subjected to hydrolysis were also analysed rheologically. Samples (30 mL) were removed from the hydrolysis reaction vessel at designated time points and inactivated by bringing the pH to 2.5 with 2 N HCl. The apparent viscosity of these samples was then measured at 16.57 rad s^{-1} at 50 ºC over 5 min. All rheological measurements were carried out in triplicate.

Light and confocal microscopy

Light microscopy was performed utilising an Olympus BX51 (Olympus Ltd., Essex, UK) running on Image Access Premium® 8 software. Samples from both the heat-denaturation and subsequent hydrolysis experiments were placed directly onto slides and aggregates were visualised and the mean length determined using the software (average of 20 samples was presented). Hydrolysis was followed in real-time by confocal laser scanning microscopy (CLSM) using a Leica TCS SP5 Confocal Scanning Laser Microscope (Leica Microsystems, Wetzler, Germany). Samples were prepared in 0.5 M phosphate buffer in an indented rubber sealed slide complete with temperature control at 50 ºC.

Statistical analysis

Analysis of variance (ANOVA) was performed through the use of Minitab 15 software (Minitab Inc., State College, PA, USA). This was followed by a Tukey test

to determine the statistical difference of means where the significance level was established for $P < 0.05$.

RESULTS AND DISCUSSION

Characterisation of heat-denatured WPI

Electrophoresis. Both SDS- and native-PAGE were utilised to characterise the nature of the inter-molecular interactions involved in the aggregation process (Fig. 1). This allowed determination as to whether the bonding occurring was through covalent or non-covalent interactions. In Fig. 1 the loss in band intensities for the major protein components in the WPI is shown by native- and non-reducing SDS-PAGE. Native-PAGE (Fig. 1 A) showed that BSA, β-lg and α-la bands decreased with increased intensity of heat-treatment. Unlike SDS-PAGE, where mobility depends primarily on molecular mass, in native-PAGE the mobility depends on both native charge and hydrodynamic size. For this reason α-la does not traverse down the gel as much as β-lg A and β-lg B and this pattern has been shown previously for WPI (*29*). During native-PAGE, the aggregates formed on heat-treatment were too large to enter the gel. This is exhibited as a loss in band intensity for the whey proteins in Lanes 5 (75 ºC x 15 min) and 6 (80 ºC x 10 min). Non-reducing SDS-PAGE (Fig. 1 B) showed a noticeable loss in the intensity of the bands for the whey proteins and the presence of high M_w aggregates particularly in Lanes 6 (75 ºC x 15 min) and 7 (80 ºC x 10 min). This shows that aggregation was not entirely due to non-covalently linked aggregates as SDS breaks hydrophobic bonds. Less whey proteins were present in the 80 ºC x 10 min heat-treated sample separated by native-PAGE (Lane 6, Fig. 1 A) when compared to non-reducing SDS-PAGE (Lane 7, Fig. 1 B). This denotes non-covalent interactions, such as hydrophobic aggregation, being present at the most intensive

heat-treatments, namely 75 ºC x 15 min and 80 ºC x 10 min. The samples on reducing SDS-PAGE (Fig 1 C) gave similar band intensities across the different heat-treatments, indicating that a high percentage of the aggregation in the WPI was the result of covalently linked di-sulphide interactions, which were reduced on treatment with 2-mercaptothanol.

Chromatography. The aggregates formed during heating of WPI represent a complex of proteins that possess differing heat resistances. RP-HPLC was used to determine whether the whey proteins were present in a native or denatured conformational state in the WPI following heat-treatment. In RP-HPLC, as the hydrophobicity changes due to thermally-induced unfolding and exposure of apolar residues, a loss in peaks corresponding to native proteins in WPI was observed. The loss in native α-la, β-lg A, β-lg B, and CMP (the proteins which constitute ~ 79 % of the total protein in the WPI) with increasing temperature and time treatment is shown in Fig. 2. CMP is a major component in WPI manufactured from cheese whey. The RP-HPLC data shows a relatively minor loss (0.6 ± 0.2 g L^{-1}) in native CMP concentration after a heat-treatment of 80 ºC x 10 min in comparison to the un-heated control. This heat resistance has been attributed to the minimal structural features of CMP which is reported to exist as an essentially disorganised macro-peptide (*31*).

The concentration of native β-lg A declined from 17.2 ± 0.2 g L^{-1} in control un-heated WPI to 4.0 ± 0.5 g L^{-1} on heat-treatment at 80 ºC x 10 min. The differences in β-lg A concentration over the range of heat-treatments and also with respect to the control un-heated solutions was significant ($P < 0.001$). Figure 2 also shows a loss in native β-lg B from 16.7 ± 0.1 g L^{-1} in control un-treated to 5.8 ± 0.4 g L^{-1} after 80 ºC x 10 min treatment. The observed greater heat lability of β-lg A compared to β-lg B is in agreement with previous work (*30*). The rate of loss in native α-la was lower than

the loss in both native β-lg variants over the heat-treatments. The concentration of native α-la decreased from 14.2 ± 0.2 g L^{-1} to 5.1 ± 0.5 g L^{-1} with the differences between the concentrations of native α-la over the various heat-treatments being significant in comparison to each other and to the control ($P < 0.001$). Overall, the greatest incremental loss in total native protein concentration on heating occurred between 65 ºC x 15 min and 70 ºC x 5 min.

Heat-denaturation of the proteins in the WPI solutions resulted in the loss of native proteins and extensive aggregation. SEC was utilised to distinguish the formation of aggregates. The aggregates produced on heat-treatment (Fig. 3 insert, Fig. 4 B and C) were greater than the exclusion limit of the guard column (~ 100 nm) or were retained during sample filtration and as a result no discernable aggregates are observed by SEC (Fig. 3). The SEC profiles demonstrate that peaks corresponding to α-la and β-lg were progressively reduced on heat-treatment. The peaks equivalent to CMP demonstrated that only 75 ºC x 15 min and WPI 80 ºC x 10 min heat-treatments significantly reduced ($P < 0.01$) CMP concentration compared to the un-heated control. During SEC elution in 20 mM sodium phosphate (pH 7) CMP exists in a multi-meric form having a molecular mass between 40 – 50 kDa (Fig. 3). Hydrophobically complexed multi-meric forms of CMP above pH 4.5 have been reported and this property has been exploited during CMP enrichment (*32*). Overall, the results from both RP-HPLC and SEC analysis indicate that CMP, in regards to concentration, is minimally involved in aggregation following heat-treatment of WPI, showing a relatively minor loss in concentration (~ 4 %) at the highest heat-treatment.

Physicochemical characteristics of heat-treated samples. The relationship between particle size (by D.v 09 and light microscopy), turbidity (OD_{550}), solubility, viscosity and surface hydrophobicity were compared as a function of heat-treatments

(see Table 1). The largest single increase in particle size (D.v 09) occurred between 65 ºC x 15 min (16.6 ± 0.2 μm) and 70 ºC x 5 min (30.9 ± 0.2 μm). This also coincided with the largest single decrease in overall native protein concentration (Fig. 2 and Table 1). The overall particle size increased from 15.9 ± 0.4 μm in the control un-heated WPI solutions to 40.2 ± 0.6 μm in the 80 ºC x 10 min WPI solutions.

In this study visualisation of aggregate formation and morphology was accomplished with light microscopy (LM). Particle length measurements for the aggregates formed through heat-treatment were carried out with the accompanying software. Similar to the D.v 09 results, these measurements also showed the largest single increase in particle length between 65 ºC x 15 min (17.5 ± 2.3 μm) and 70 ºC x 5 min (32.9 ± 2.0 μm) heat-treatments of WPI. Figure 4 (A-C) shows the increase in the extent of aggregation from un-heated control (Fig. 4 A) to 80 ºC x 10 min (Fig. 4 C). Heat-treatment above 65 ºC x 15 min resulted in white solutions (results not shown) and a corresponding increase in turbidity (OD_{550}), with the greatest increase occurring after heating at 80 ºC x 10 min (Table 1). Heat-induced unfolding and subsequent aggregation led to insolubilisation, resulting in a reduction in solubility from 90 ± 1 % in the control un-heated solution to 31 ± 2 % solubility on heating at 80 ºC x 10. The largest single decrease in solubility occurred for WPI heated at 70 ºC x 15 min (71 ± 2 %) and 75 ºC x 5 min (56 ± 2 %). The high extent of insolubilisation at heat-treatments greater than 70 ºC for 5 min meant that the solution of denatured WPI could only be kept in suspension through stirring. Rotational rheological analysis of the heat denatured WPI solutions (Table 1) demonstrated an increase in apparent viscosity from 15.5 ± 0.4 mPa s^{-1} for un-heated control to 247.1 ± 5.0 mPa s^{-1} for the 80 ºC x 10 min heated samples. The largest single increase in

viscosity was observed to occur on heating between 70 ºC x 15 min (20.4 ± 0.9 mPa s^{-1}) and 75 ºC x 5 min (118.4 ± 3.1 mPa s^{-1}).

The surface hydrophobicity (SH) of the protein solutions after dialysis was expressed as μg SDS bound per 500 μg protein (Table 1). The disruption of hydrophobic interactions through binding of SDS facilitates movement of the cationic methylene blue dye into the chloroform phase (*33*). The SH of WPI increased by a factor of 4.75 on heating at 75 ºC x 15 min (see Table 1). Interestingly, the SH decreased slightly to 70.11 ± 2.01 μg SDS / 500 μg protein after 80 ºC x 10 min treatment of the WPI solutions, although this decrease was not significant ($P < 0.05$). These results were in agreement with the general trend of a previous study on a WPI (*27*). A loss in protein structure would theoretically increase the number of hydrophobic residues accessible (*3*) to the SDS-complex. A previous study demonstrated that during extensive aggregation SH may be expected to decrease if aggregates were linked via non-covalent hydrophobic interactions (*34*). However, the native- and SDS-PAGE results herein showed that a substantial degree of the aggregated material appeared to be linked covalently via di-sulphide linkages.

Rationale for physicochemical changes. Heating WPI at different temperature / time combinations led to the formation of aggregates giving functionally diverse solutions / suspensions from the un-heated control. Heating the WPI resulted in white, high turbidity solutions, with increased insolubility and viscosity. The reason for the large increase in viscosity may be due to the increased particle size and the heat-treated insoluble whey proteins binding more water, which leads to an increase in apparent viscosity (*35*). The large aggregates, up to 40.2 ± 0.6 μm (D. v 09) were morphologically irregular when visualised by both light and confocal microscopy (Fig 4C).

Many contributing factors may hasten the onset of protein aggregation. According to the three stage denaturation model (*36*), the existence of some denatured protein prior to heat-treatment allows for the propagation of aggregation upon the application of heat. In our study, the presence of some particles having particle size values of 15.9 ± 0.4 μm (D. v 09) in the un-heated control may have influenced the formation of larger aggregates (> 30 μm) on heating at temperatures greater than 65 ºC. Furthermore, since the WPI preparation was obtained from rennet whey, the presence of relatively high amounts of calcium (0.62 g 100 g^{-1} dried wt.) determined by the accepted IDF / ISO method (*37*), may induce electrostatic screening and accelerate the aggregation process (*5, 38, 39*). In addition, the use of a relatively high protein concentration (100 g L^{-1} protein) herein may also have promoted aggregation (*4, 40*).

Enzymatic hydrolysis of control and heat-denatured WPI

Influence on reaction rate. An increase in the rate of hydrolysis was observed in certain WPI solutions which were subjected to heat-treatment (Fig. 5). Previous studies have demonstrated improved reaction rates as a consequence of pre-treatment of whey protein substrates (*12, 41*). The time to reach a DH of 5 % for a WPI solution subjected to pre-hydrolysis heat treatment of 80 ºC x 10 min (53 ± 7 min) was approximately half that of a un-heated control WPI solution (115 ± 4 min). Heating WPI solutions (100 g L^{-1}) to 80 ºC for 10 min followed by hydrolysis, at an E:S of 1:100, gave an equivalent reaction time to un-heated control WPI solutions (100 g L^{-1}) hydrolysed at an E:S of 1.5:100 (Fig. 5 insert). Solutions which were subjected to pre-hydrolysis treatment at temperatures ≥ 75 ºC x 5 min had a statistically significant increased rate of hydrolysis ($P < 0.001$) compared to the un-heated control. A 72 %

loss in native protein concentration in WPI solutions heated at 80 ºC for 10 min compared to the un-heated control resulted in a 54 % decrease in hydrolysis time to achieve a DH of 5 % (Fig. 5). Those samples subjected to heat-treatments; 60 ºC x 15 min, 65 ºC x 5 min, 65 ºC x 15 min, 70 ºC x 5 min, and 70 ºC x 15 min displayed similar hydrolysis curves to control un-treated WPI and were not statistically different from the control hydrolysis reaction using the comparative Tukey test ($P < 0.05$). Therefore, the polymerisation of the whey proteins into aggregates ($\geq$ 36.9 μm) along with a total loss of native protein $\geq$ 41 % presented a conformation which was beneficial to enzymatic hydrolysis with Corolase® PP.

Heat-denaturation of the WPI presumably exposes previously buried hydrolytic cleavage sites through structural changes in the whey proteins. Protein denaturation, which reduces tertiary and quaternary structure, reduces the ability of the protein to internalise and protect certain residues from hydrolytic cleavage (*42*). Denaturation of the polypeptide chain can result in a marked increase in the number of peptide bonds available for reaction (*28*). Adsorption and diffusion phenomena may also affect the rate of hydrolysis in irreversibly denatured protein (*43*) where large aggregates possess a much lower diffusion coefficient than the enzyme. However, this was minimised for our hydrolysis experiments which were performed under moderate stirring.

Physicochemical characteristics of hydrolysates. Table 2 summarises the physicochemical characteristics of the un-heat treated control, and the treatments showing the greatest increase in reaction rate (namely WPI 75 ºC x 5 min, 75 ºC x 15 min, and 80 ºC x 10 min) following hydrolysis with Corolase® PP. On hydrolysis to 5 %DH with Corolase® PP, particle size and turbidity were reduced in all the WPI solutions. This was especially the case for solutions subjected to the most extreme

heat-treatments. The D.v 09 results show a 45.7 % decrease in particle size on hydrolysis to 5 % DH of WPI 80 ºC x 10 min (Table 2) compared to the un-hydolysed 80 ºC x 10 min solution (Table 1). Table 2 also shows that hydrolysis of the most extensively heat-denatured WPI solutions, namely 75 ºC x 5 min, 75 ºC x 15 min, and 80 ºC x 10 min, resulted in an increase in solubility of the solution at 5 % DH compared to the respective un-hydrolysed samples (Table 1). The increase in solubility was greatest in the WPI 80 ºC x 10 min hydrolysates, which showed an increase in solubility of 9 ± 4 % compared to the un-hydrolysed WPI 80 ºC x 10 min heat-treated substrate prior to hydrolysis.

The changes in apparent viscosity taking place during hydrolysis of un-heated control and heat-treated solutions of WPI samples was monitored though rotational rheological analysis. Over the course of the hydrolysis reaction the mean apparent viscosity of the WPI non-heat treated control solution decreased; from 1.61 ± 0.69 x 10^{-2} Pa/s at 0.2 % DH to 1.39 ± 0.51 x 10^{-2} Pa/s at 5 %DH. On the other hand, the apparent viscosity of the WPI 75 ºC x 5 min, 75 ºC x 15 min, and 80 ºC x 10 min solutions decreased significantly ($P < 0.001$) at the 5 %DH level (Table 2) compared to their un-hydrolysed equivalents (Table 1).

SH of the hydrolysates (Table 2) showed an increase in comparison to the respective un-hydrolysed starting solutions (Table 1). The greatest mean percentage increase in SH was 16.5 % in the 80 ºC x 10 min hydrolysate (83.97 ± 2.59 μg SDS / 500 μg protein) compared to the un-hydrolysed 80 ºC x 10 min solution (70.11 ± 2.01 μg SDS / 500 μg protein). This trend is in agreement with previous work on a heat-denatured WPI (*42*). However, peptide-protein hydrophobic interactions might have influenced the determinations.

Microscopic analysis during the course of hydrolysis. Visualisation of the structural changes occurring in the WPI as a result enzymatic hydrolysis was achieved through microscopic analysis. In the solutions which were heat-treated prior to enzymatic hydrolysis it was possible to discern the destabilisation of aggregates by LM. Figure 4 D-F shows the changes in WPI heat-treated at 80 ºC x 10 min during subsequent hydrolysis. Fig. 4 E shows the solution at 4.7 % DH (t_{+50} min), where a noticeable reduction in the extent of aggregation is observed compared to Fig 4 D (t_{+2} min). Allowing the enzymatic reaction to continue to 8.1 % DH (Fig 4 F) reduced the extent of the aggregation leading to increased optical clarification. This was demonstrated by the fact that the turbidity (0.21 ± 0.03, OD_{550}) at 8.1 % DH of the 80 ºC x 10 min heat-treated solution was similar to the turbidity (0.17 ± 0.02, OD_{550}) of a 65 ºC x 5 min heat-treated solution pre-hydrolysis.

Hydrolysis was also followed *in situ* using CLSM where the WPI solution (100 g L^{-1} protein) was stained with acridine orange. A previous study (*44*) utilised microscopy (TEM) to visualise the structural differences of β-lg pre- and post-hydrolysis with pepsin. In our study, a novel method for determining aggregate disintegration was presented through the use of a sealed-cell in CLSM which allowed for the hydrolysis reaction to be followed 'real-time'. From the CLSM images (Fig. 6) the formation of morphologically irregular aggregates is apparent in the hydrolysed WPI subjected to pre-heating at 80 ºC for 10 min. In Fig. 6 A the aggregates appear to be quite regularly distributed after 5 min of hydrolysis. However, large gaps began to appear as hydrolysis proceeds and an aggregate network began to form (Fig. 6B and 6C) as aggregate size was reduced (Table 2) from 40.17 ± 0.63 μm (Table 1) at time zero to 22.10 ± 0.47 μm after 50 min hydrolysis (results not shown).

Conclusion. Aggregation of WPI by thermal pre-treatment allowed for improved susceptibility to hydrolysis with Corolase® PP. This improvement was most marked in WPI solutions which had been subjected to a heat-treatment ≥ 75 ºC for 5 min. This study showed how different heat-treatments changed the structural characteristics of the substrate at the individual protein level along with the associated changes in their functional attributes, e.g. solubility. Utilising various analytical techniques allowed for the quantification and visualisation of the changes taking place both as a result of heat-treatment and during the course of subsequent hydrolysis. The use of both light and confocal microscopy offered a new approach for monitoring enzymatic hydrolysis of food proteins.

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Figure 1. Electrophoresis of 100 g L^{-1} protein WPI solutions (pH 6.4) subjected to different heat treatments: (A) Native-PAGE where; Lane (1) Unheated control, (2) 60ºC for 15min, (3) 65ºC for 15min, (4) 70ºC for 15min, (5) 75ºC for 15min, (6) 80ºC for 10min. (B) Non-reducing SDS-PAGE and (C) Reducing SDS-PAGE. For both SDS-PAGE gels; Lane (1) marker, (2) Unheated control, (3) 60ºC for 15min, (4) 65ºC for 15min, (5) 70ºC for 15min, (6) 75ºC for 15min, (7) 80ºC for 10min, (8) marker.

Figure 2. Native protein composition of 100 g L^{-1} untreated control and heat treated WPI solutions subjected to different temperature (ºC) x time (min) treatments determined by reversed phase-HPLC (sample mean ± SD, n = 3).

Figure 3. Reversed-phase chromatography of WPI (100 g L^{-1} protein) solutions subjected to heat treatments. Where; (A) represents WPI unheated control, (B) WPI 75 ºC for 5 min, (C) WPI 75 ºC for 15 min and (D) WPI 80 ºC for 10 min. Eluate was measured at 214 nm.

Figure 3. Size exclusion chromatography of WPI (100 g L^{-1} protein) solutions subjected to heat treatments. Where; ; represents WPI unheated control, ; intermediate heat treatments and, ; WPI 80 ºC for 10 min. Samples were eluted with 20 mM Na-Phosphate buffer. Insert is a confocal laser scanning microscopy image of an isolated WPI aggregate formed after heat treatment (75 ºC for 5 min), where the black bar represents 15 μm.

Figure 4. Light microscope images of whey protein isolate (WPI) solutions (100 g L^{-1} protein): (A) un-heated control, (B) heat-treated 70 ºC for 15 min, and (C) 80 ºC for

10 min. Solution of 80 ºC for 10 min treated WPI subsequently hydrolysed with Corolase® PP (1:100 E:S) pH 8 at (D) 2 min (0.4 %DH), (E) 50 min (4.7 %DH) and (F) 130 min (8.1 %DH).

Figure 5. Hydrolysis profiles for whey protein isolate (100 g L^{-1} protein, pH 8) unheated control and heat treated solutions to a degree of hydrolysis (DH) of 5 % with Corolase® PP (1:100 E:S) as obtained using the pH-stat method. Where; ◇ represents control, ◆ represents 75 ºC for 5 min, □ represents 75 ºC for 15 min, and ■ represents 80 ºC for 10 min. Insert is of 100 g L^{-1} protein WPI solutions which were subjected to hydrolysis with varying concentrations of enzyme (◇) and the time to reach a DH of 5 % was measured.

Figure 6. Confocal Laser Scanning Microscopy (CLSM) images of whey protein isolate (100 g L^{-1} protein) subjected to heating at 80 ºC for 10 min, stained with acridine orange, and subsequently hydrolysed with Corolase® PP (1:100 E:S) at (A) 5 min (0.9 %DH), (B) 30 min (3.4 %DH), and (C) 56 min (5 %DH).

Table 1. Physicochemical characteristics of whey protein isolate (100 g L^{-1} protein, pH 6.4) solutions subjected to varying temp (ºC) x time (min) heat-treatments.

Test sample	Particle size				Turbidity	Solubility	Apparent Viscosity	Hydrophobicity
	D.v. 09 (μm)	D.v. 05 (μm)	D.v. 01 (μm)	LM (μm)	OD_{550} *a*	(%)	(mPa s^{-1})	*b*
WPI Control	15.9 ± 0.4^{X}	5.3 ± 0.3^{X}	0.5 ± 0.3^{X}	*c*	0.04 ± 0.01	90 ± 1	15.5 ± 0.4^{X}	15.53 ± 1.27^{X}
WPI 60ºC x 15min	$16.4 \pm 0.3^{X,Y}$	5.5 ± 0.4^{X}	0.8 ± 0.2^{X}	16.2 ± 1.8^{X}	0.11 ± 0.02	84 ± 1^{X}	$15.6 \pm 0.8^{X,Y}$	$17.17 \pm 1.50^{X,Y}$
WPI 65ºC x 5min	$16.3 \pm 0.5^{X,Y}$	5.7 ± 0.2^{X}	0.6 ± 0.4^{X}	17.4 ± 1.6^{X}	0.17 ± 0.02	85 ± 1^{X}	$15.6 \pm 0.9^{X,Y}$	$20.37 \pm 1.94^{Y,Z}$
WPI 65ºC x 15min	16.6 ± 0.2^{Y}	5.6 ± 0.3^{X}	0.7 ± 0.2^{X}	17.5 ± 2.3^{X}	0.29 ± 0.07	80 ± 2^{Y}	$16.1 \pm 0.9^{X,Y}$	23.53 ± 1.45^{Z}
WPI 70ºC x 5min	30.9 ± 0.2	10.9 ± 0.1^{Y}	1.4 ± 0.3^{Y}	32.9 ± 2.0^{Y}	0.41 ± 0.05	77 ± 2^{Y}	16.9 ± 0.8^{Y}	36.71 ± 2.40
WPI 70ºC x 15min	33.3 ± 0.1	11.2 ± 0.4^{Y}	1.4 ± 0.4^{Y}	$35.0 \pm 1.8^{Y,Z}$	0.65 ± 0.09	71 ± 2	20.4 ± 0.9	51.97 ± 3.30
WPI 75ºC x 5min	36.8 ± 0.4	13.3 ± 0.1^{Z}	1.6 ± 0.2^{Y}	38.6 ± 2.5^{Z}	0.95 ± 0.13	56 ± 2	118.4 ± 3.1	65.15 ± 1.91
WPI 75ºC x 15min	38.9 ± 0.7	13.8 ± 0.5^{Z}	$1.9 \pm 0.1^{Y,Z}$	44.6 ± 3.2^{W}	1.86 ± 0.20	43 ± 3	214.9 ± 6.7	73.69 ± 3.03^{W}
WPI 80ºC x 10min	40.2 ± 0.6	14.8 ± 0.2	2.2 ± 0.3^{Z}	45.7 ± 4.7^{W}	>2	31 ± 2	247.1 ± 5.0	70.11 ± 2.01^{W}

Same letters within a column; x, y, z,… indicate insignificant differences ($P > 0.05$).

a Optical density at 550 nm (OD_{550}), protein solutions diluted to 2.5 g L^{-1} for absorbance ≤ 1.

b Expressed as μg SDS bound per 500 μg of protein.

c No aggregates accurately discerned with light microscopy (LM) ≤ 600x.

Error! Not a valid link.

Table 2. Physicochemical characteristics of whey protein isolate (100 g L^{-1} protein) and hydrolysed, to a degree of hydrolysis of 5 % with Corolase® PP (1:100 E:S)**Error! Not a valid link.**, un-heated control and heat-treated solutions. **Error! Not a valid link.**

Test sample	Particle size				Turbidity	Solubility	Apparent Viscosity	Hydrophobicity
	D.v. 09 (μm)	D.v. 05 (μm)	D.v. 01 (μm)	LM (μm)	OD_{550} *a*	(%)	(mPa s^{-1})	*b*
WPI Control	15.9 ± 0.4	5.3 ± 0.3^{X}	0.5 ± 0.3^{X}	*c*	0.04 ± 0.01^{X}	90 ± 1^{X}	(15.5 ± 0.4^{X}) *d* 16.1 ± 0.2^{X}	15.53 ± 1.27^{X}
Hydrolysates								
WPI Control	13.5 ± 0.5	4.8 ± 0.3^{X}	0.7 ± 0.2^{X}	*c*	0.03 ± 0.01^{X}	92 ± 2^{X}	13.9 ± 5.1^{X}	17.49 ± 2.31^{X}
WPI 75ºC x 5min	20.3 ± 0.6^{X}	6.7 ± 0.4^{Y}	1.0 ± 0.4^{X,Y}	21.4 ± 2.8^{X}	0.83 ± 0.08^{Y}	59 ± 2	97.2 ± 3.5^{Y}	73.06 ± 2.63
WPI 75ºC x 15min	20.2 ± 0.4^{X}	7.0 ± 0.2^{Y}	1.1 ± 0.5^{X,Y}	25.2 ± 4.2^{X}	0.97 ± 0.25^{Y,Z}	50 ± 3	105.8 ± 7.9^{Y,Z}	81.72 ± 1.66^{Y}
WPI 80ºC x 10min	21.8 ± 0.5	7.1 ± 0.3^{Y}	1.2 ± 0.3^{Y}	23.3 ± 7.3^{X}	1.26 ± 0.24^{Z}	42 ± 3	113.1 ± 7.4^{Z}	83.97 ± 2.59^{Y}

a Optical density at 550 nm (OD_{550}), protein solutions diluted to 2.5 g L^{-1} for absorbance ≤ 1.

b Expressed as μg SDS bound per 500 μg of protein.

c No aggregates accurately discerned with light microscopy (LM) ≤ 600x.

d Apparent viscosity determined at pH 6.4.

Figure 1 A

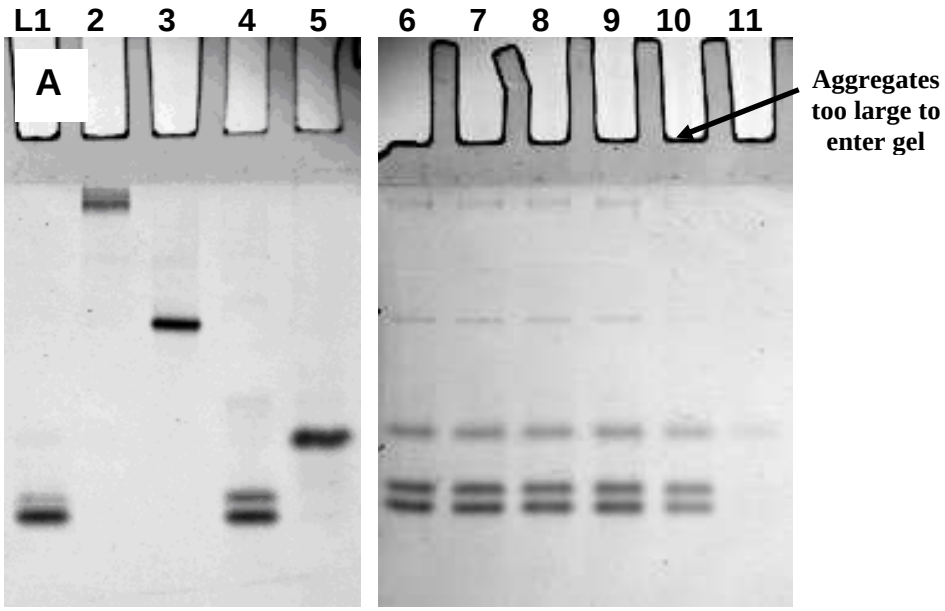


Figure 1 B

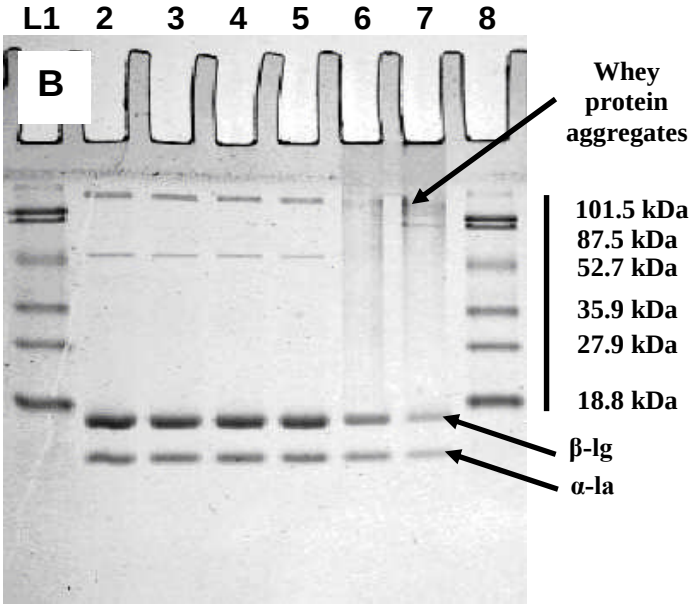


Figure 1 C

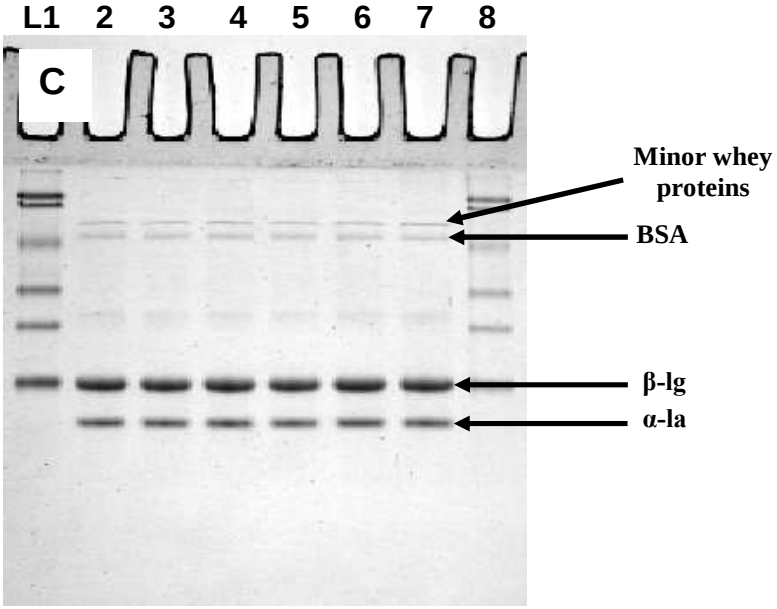


Figure 2

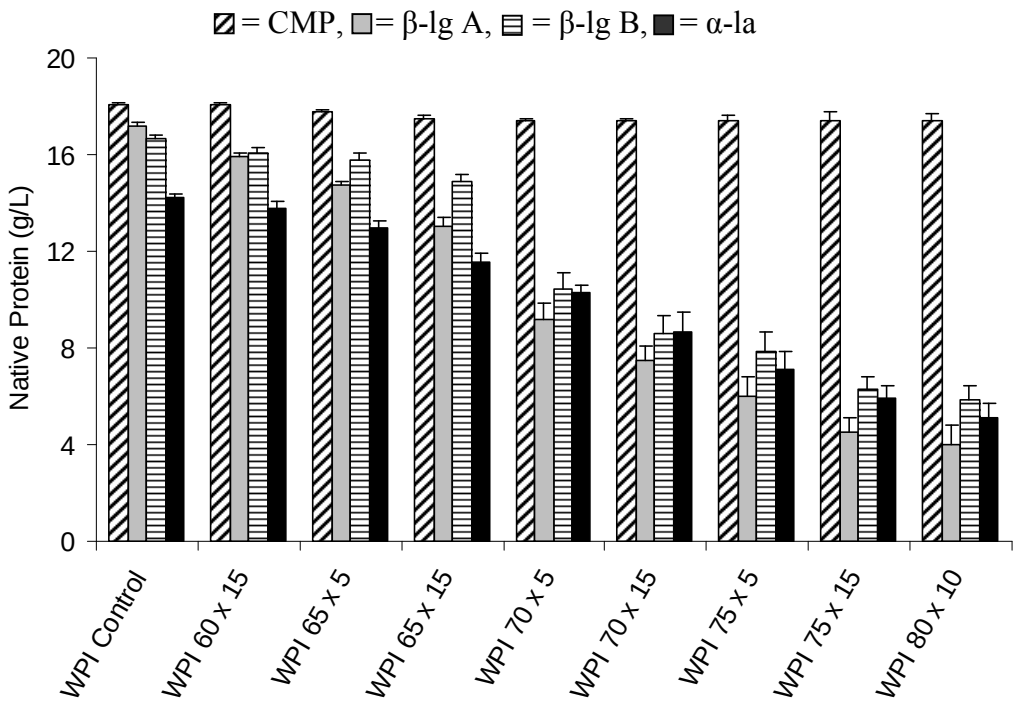
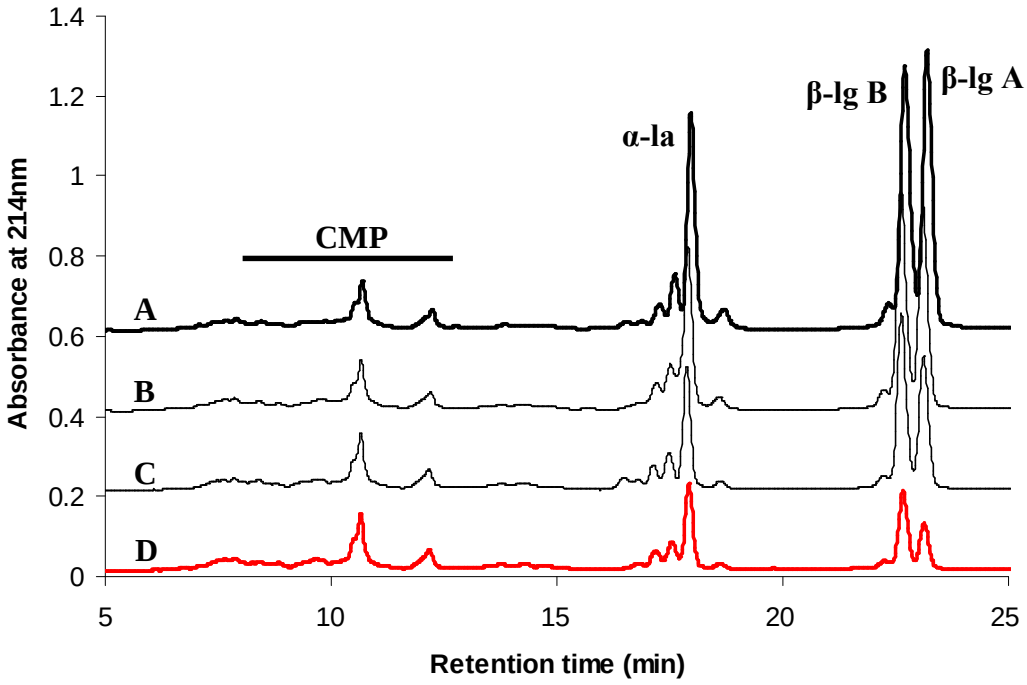
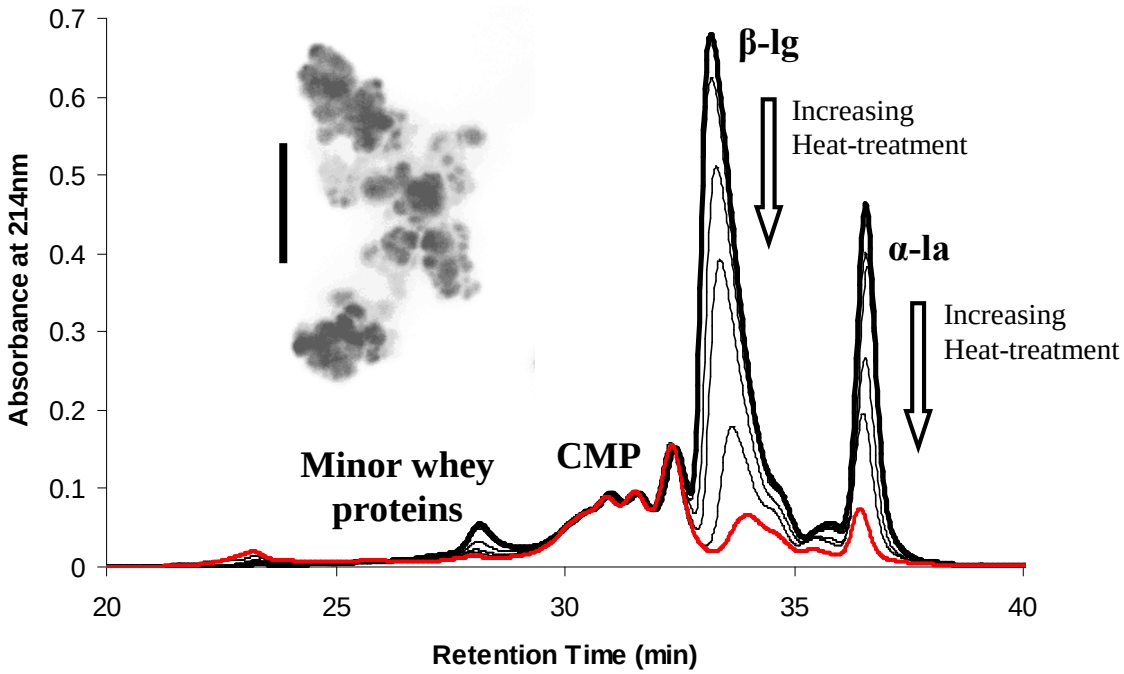


Figure 3 (intended for colour reproduction on-line and in print)




0.7
0.6
0.5
0.4
0.3
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0
Absorbance at 214nm
20
25
30
35
40
Retention Time (min)
β-lg
Increasing
Heat-treatment
α-la
Increasing
Heat-treatment
Minor whey
proteins
CMP

Figure 4

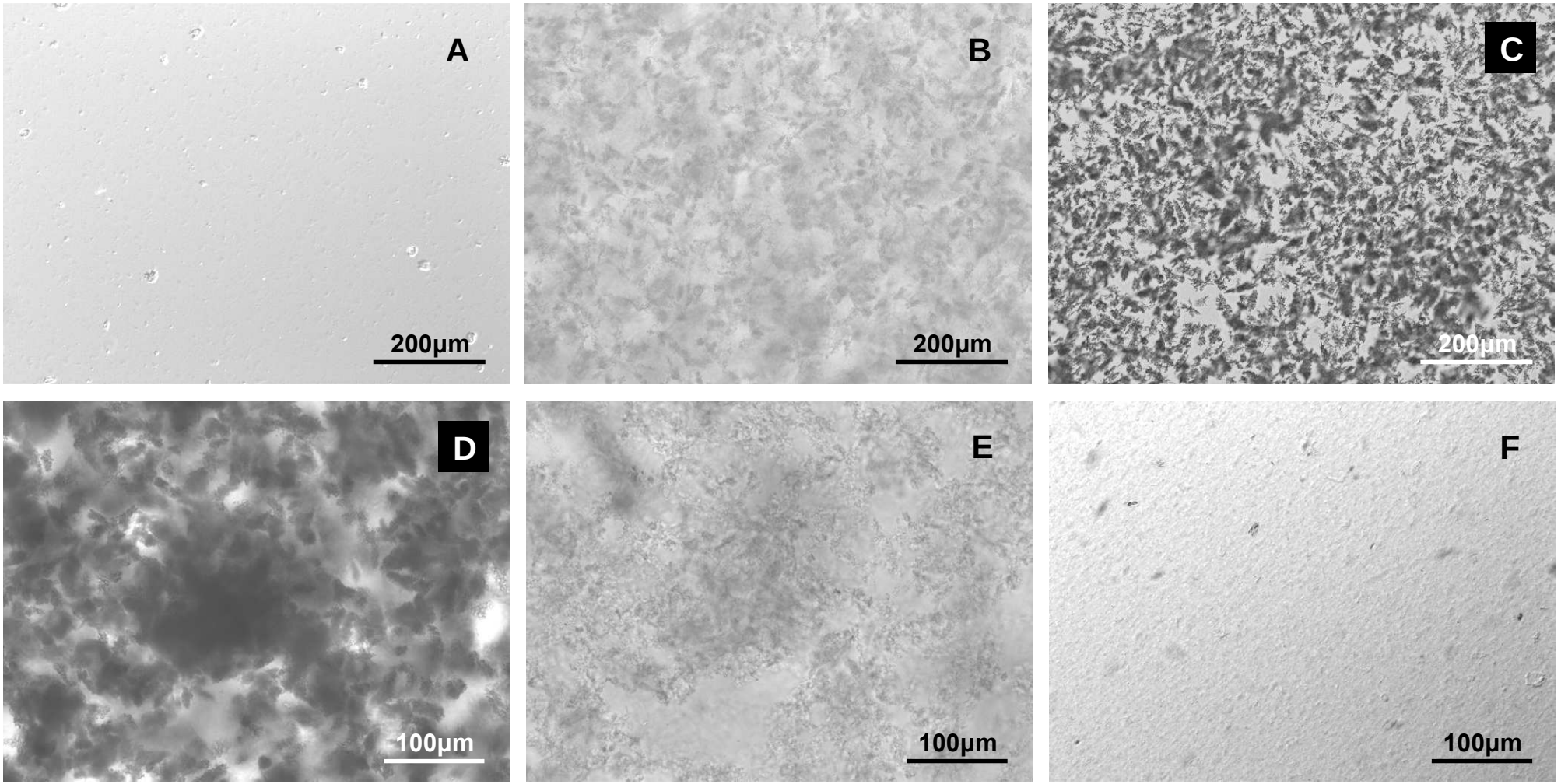

Figure 5

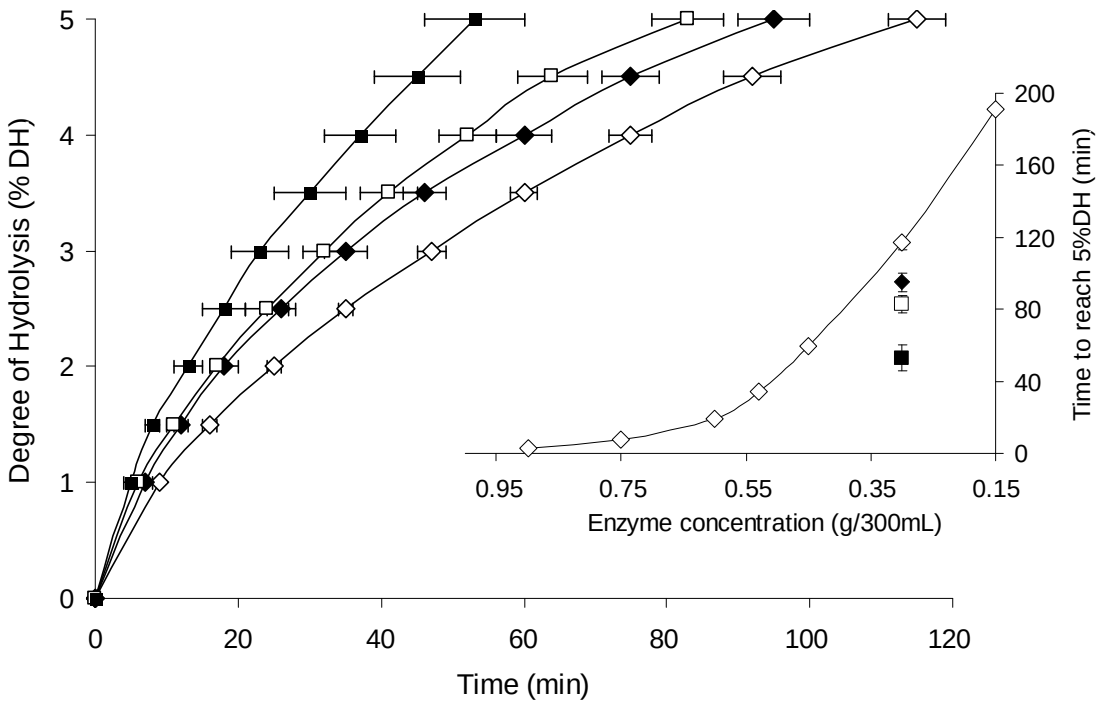
Degree of Hydrolysis (% DH)
Time (min)
0
1
2
3
4
5
0
20
40
60
80
100
120
Time to reach 5%DH (min)
Enzyme concentration (g/300mL)
0.95
0.75
0.55
0.35
0.15
0
40
80
120
160
200

Figure 6 (intended for colour reproduction on-line and in print)