JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Enzymatic Hydrolysis of Heat-Induced Aggregates of Whey Protein Isolate

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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. Both heated (60 °C, 15 min; 65 °C, 5 and 15 min; 70 °C, 5 and 15 min, 75 °C, 5 and 15 min; 80 °C, 10 min) and unheated WPI solutions (100 g L^{-1} protein) were incubated with a commercial proteolytic enzyme preparation, Corolase PP, until they reached a target degree of hydrolysis (DH) of 5%. WPI solutions on heating were characterized by large aggregate formation, higher viscosity, and surface hydrophobicity and hydrolyzed more rapidly ($P < 0.001$) than the unheated. The whey proteins exhibited differences in their susceptibility to hydrolysis. Both viscosity and surface hydrophobicity along with insolubility declined as hydrolysis progressed. However, microstructural changes observed by light and confocal laser scanning microscopy (CLSM) provided insights to suggest that aggregate size and porosity may be complementary to denaturation in promoting faster enzymatic hydrolysis. This could be clearly observed in the course of aggregate disintegration, gel network breakdown, and improved solution clarification.

KEYWORDS: *enzymatic hydrolysis, whey protein isolate, denaturation, aggregation*

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 unfolding, 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 °C.[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 minimize exposure of hydrophobic (apolar) residues to solvent.

Extended exposure to temperatures >60 °C can irreversibly affect the solubility of whey proteins[2] and change the relative hydrophobicity at the protein surface leading to exposure of nonpolar hydrophobic residues thereby increasing hydrophobic attraction. Hydrophobic interactions are reported to increase with increasing temperature up to ~70 °C, after which they diminish.[1] Exposure of sulfhydryl 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 °C, free cysteine residues in β-lg and BSA lead to disulfide 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 disulfide 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 disulfide bond reduction.[15] For example, native β-lg is resistant to hydrolysis by pepsin, however, heat treatment of β-lg at 82 °C results in peptic hydrolysis.[12] The exposure of hydrophobic residues can lead to greater reactivity,[4] and heat-denatured whey proteins can be readily solubilized during hydrolysis.[16]

It is noteworthy that not all thermal treatments result in an increase in hydrolytic susceptibility.[17,18] Preheating of α-la at 95 °C for 10 min resulted in reduced hydrolysis activity with Pronase, trypsin, and pancreatin activities.[17] This highlights the interdependence of pretreatment 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 physicochemical

Received: December 19, 2011
Revised: April 19, 2012
Accepted: April 25, 2012
Published: April 26, 2012

dx.doi.org/10.1021/jf205213n | *J. Agric. Food Chem.* 2012, 60, 4895−4904

properties, the function of prehydrolysis 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 characterize 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 [$N \times 6.38$,[19]] comprising 56.5% β-lactoglobulin, 14.3% α-lactalbumin, 10.3% glycosylated caseinomacropeptide (CMP), 8.0% nonglycosylated CMP, 1.3% lactoferrin (LF), and 1.7% bovine serum albumin (BSA) according to the manufacturer. 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 weight cutoff 3500 Da) 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 to 80 °C for selected time periods, i.e., 5, 10, and 15 min. The specific temperature (°C) × time (min) treatments were as follows; 60 × 15, 65 × 5, 65 × 15, 70 × 5, 70 × 15, 75 × 5, 75 × 15, and 80 × 10. Samples were solubilized in deionized water and allowed to hydrate overnight at 4 °C. Heat treatments were performed in triplicate in a thermostatically controlled water bath. At the end of each treatment solutions were placed in an ice/water bath. Freeze-dried powders were stored in a cool low moisture environment.

Chromatographic Characterization of Control and Heated-Treated WPI Solutions. High performance liquid chromatography (HPLC) was carried out using a Waters 2695 separation module and 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 × 4.6 mm, column (GE Healthcare, Buckinghamshire, U.K.). Solvent A was 0.1% trifluoroacetic acid (TFA) in Milli-Q water, and solvent B was 90% HPLC-grade acetonitrile (MeCN) containing 0.1% TFA in Milli-Q 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,[22] 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 × 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 Milli-Q water (2.5 g L^{-1} solutions) prefiltered through 0.45 μm low protein binding membrane filters (Sartorius Stedim Biotech GmbH, Germany) and 20 μL applied to the column. The column elution was monitored at 214 nm, and all solvents were filtered under vacuum through 0.45 μm high velocity filters (Millipore (UK) Ltd., Durham, U.K.).

Electrophoresis. Sodium dodecyl sulfate 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 nonreducing 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 °C 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 persulfate 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. Standards of BSA, β-lg A, β-lg, and α-la were from Sigma, with IgG from Upfront Chromatography (Upfront Chromatography A/S, Copenhagen, Denmark).

For both SDS−PAGE 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 volume), and gels were destained in an acetic acid:isopropanol:H_2O solution (3:10:17 by volume). 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, U.K.) 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 × 80 × 180 mm) with a maximum sample capacity of 120 mL. The solutions were diluted in deionized water to give a laser obscuration of between 14 and 19% for optimal detection. Each sample was analyzed in triplicate, and the D. v 01, D. v 05, and D. v 09 were 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. Similarly, the D. v 01 is the diameter where 10% possess a diameter less than or equal to the stated value, and in D. v 05 50% of the measured particles possess a diameter above and 50% below 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 dialyzed (MWCO 3.5 kDa) against sodium dihydrogen phosphate dehydrate buffer (1:25) for 24 h at 20 °C. Mixtures of 0.5 mL of dialysate, 2.5 mL of methylene blue, and 10 mL of chloroform were centrifuged at 2500*g* for 5 min. The extinction of the chloroform phase was assessed at a wavelength of 655 nm according to the method of Hiller and Lorenzen.[27] Chloroform served as the solvent blank, and a calibration curve was created for 0−100 μg of SDS. Surface hydrophobicity (SH) was then determined

according to eq 1. Duplicate analysis of each heat-treated and hydrolysate replicate was carried out.

$$\text{SH } (\mu\text{g of SDS}/500\ \mu\text{g of protein}) = (A_{655} - 0.0392)/0.0178 \quad (1)$$

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 of deionized 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, U.K.). For turbidity two aliquots of each replicate were analyzed.

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 M HCl. All test samples were centrifuged at 1330*g* for 30 min at 20 °C in an Eppendorf 5810 R centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was removed and filtered through Whatman No. 1 (Whatman International Ltd., Kent, U.K.) cellulose filter paper, after which protein concentration was determined by Kjeldahl ($N \times 6.38$). 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). For solubility experiments duplicate analysis of each heat-treated and hydrolysate replicate was carried out.

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]

$$\text{DH}\% = 100BN_B(1/\alpha)(1/\text{MP})(1/h_{tot}) \quad (2)$$

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 °C, MP is the mass of protein (g), and h_{tot} is the total number of peptide bonds given in mequiv g^{-1} ($N \times f_N$). 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 hydrolyzed 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 °C 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 M NaOH using a Metrohm 842 Titrando dosing unit (Metrohm Ltd., Herisau, Switzerland), and the reaction was agitated utilizing an overhead stirrer at 300 rpm. The reaction was terminated by heating the enzyme at 85 °C for 20 min. All hydrolysis experiments were conducted in triplicate.

The end-point DH was also measured by the reaction of liberated α-amino groups (α-NH_2) with 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS) through modification of the method of Adler-Nissen.[29] Using L-leucine as a standard, 2−28 mg L^{-1} amino nitrogen solutions were prepared as a standard curve. Test samples (250 μL of 3 mg mL^{-1} protein solutions made up in 1% (w/v) SDS) were added to 2 mL of 0.2125 M sodium phosphate buffer (pH 8.2). 250 μL of 1% (w/v) SDS was used as a blank. To this solution 2 mL of TNBS (1 mg mL^{-1}) was added and reacted at 50 °C for 60 min (all light excluded). The reaction was terminated by addition of 4 mL of 0.1 M HCl. Solutions were allowed to cool (30 min), and absorbance was read at 340 nm on a UV−visible spectrophotometer previously described. The DH values obtained were compared to the pH-stat determined DH. Duplicate reactions of each of the three replicates of hydrolysis were performed.

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, U.K.), 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, U.K.). Samples (28 g, 100 g L^{-1} protein) were first presheared at 16.57 rad s^{-1} for 1 min and equilibrated for 1 min at 25 °C. Samples were then sheared at a constant value of 16.57 rad s^{-1}, and the viscosity was measured after exactly 5 min of shearing. Rheological measurements of heat denaturation of WPI were taken at intervals between 25 and 80 °C. Unheated control and heat-treated samples which were subjected to hydrolysis were also analyzed 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 M HCl. The apparent viscosity of these samples was then measured at 16.57 rad s^{-1} at 50 °C over 5 min. All rheological measurements were carried out in triplicate on heat-treated and hydrolyzed replicates.

Light and Confocal Microscopy. Light microscopy was performed utilizing an Olympus BX51 (Olympus Ltd., Essex, U.K.) running on Image Access Premium 8 software. Samples from both the heat denaturation and subsequent hydrolysis experiments were placed directly onto slides, aggregates were visualized, and the mean length was 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 °C.

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

Characterization of Heat-Denatured WPI. *Electrophoresis.* Both SDS−PAGE and native-PAGE were utilized to characterize the nature of the intermolecular interactions involved in the aggregation process (Figure 1). This allowed determination as to whether the bonding occurring was through covalent or noncovalent interactions. In Figure 1 the loss in band intensities for the major protein components in the WPI is shown by native-PAGE and nonreducing SDS−PAGE. Native-PAGE (Figure 1A) 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.[30] 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 10 (75 °C × 15 min) and 11 (80 °C × 10 min). Nonreducing SDS−PAGE (Figure 1B) 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 °C × 15 min) and 7 (80 °C × 10 min). This shows that aggregation was not entirely due to noncovalently linked aggregates as SDS breaks hydrophobic bonds. Less whey protein was present in the 80 °C × 10 min heat-treated sample separated by native-PAGE (lane 11, Figure 1A) when compared to nonreducing SDS−PAGE (lane 7, Figure 1B). This denotes noncovalent interactions, such as hydrophobic aggregation, being present at the most intensive heat treatments, namely, 75 °C × 15 min and 80 °C × 10 min. The samples on reducing SDS−PAGE (Figure 1C) gave similar band intensities across the different heat treatments, indicating that a high percentage of the aggregation in the WPI was the

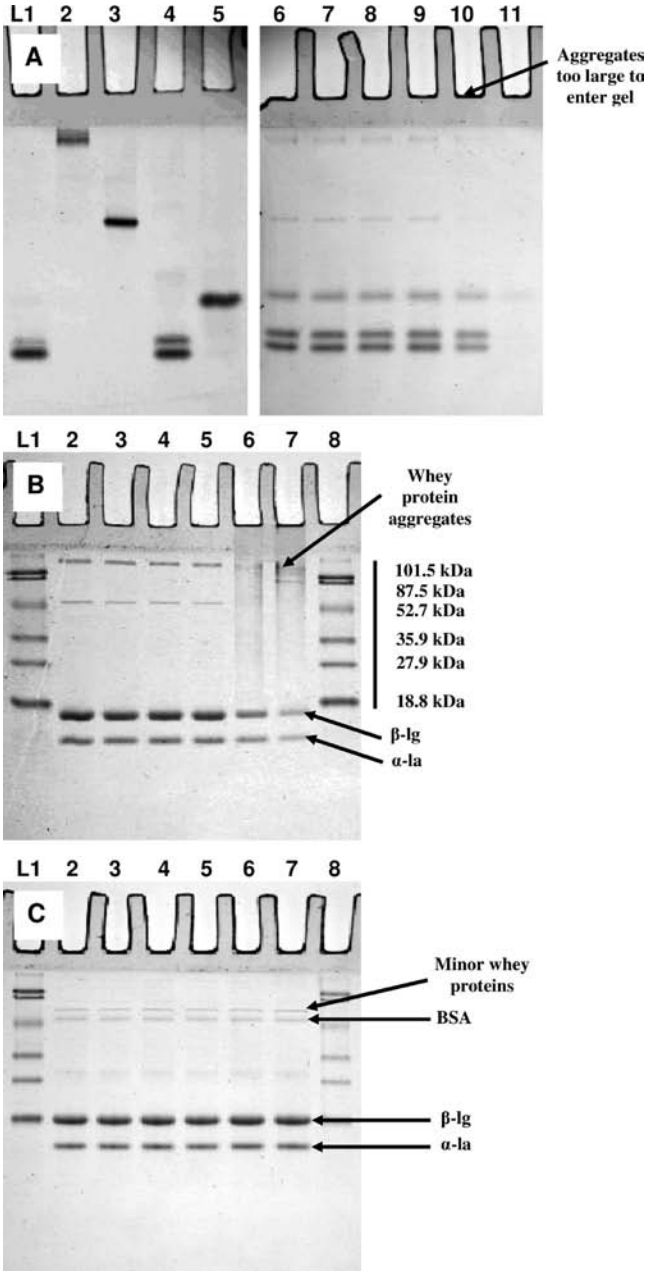

Figure 1. Electrophoresis of 100 g L^{-1} protein WPI solutions (pH 6.4) subjected to different heat treatments: (A) native-PAGE, lane (1) β-lg A, (2) IgG, (3) BSA, (4) β-lg, (5) α-la, (6) unheated control, (7) 60 °C for 15 min, (8) 65 °C for 15 min, (9) 70 °C for 15 min, (10) 75 °C for 15 min, (11) 80 °C for 10 min; (B) nonreducing SDS−PAGE; and (C) reducing SDS−PAGE. For both SDS−PAGE gels: lane (1) marker, (2) unheated control, (3) 60 °C for 15 min, (4) 65 °C for 15 min, (5) 70 °C for 15 min, (6) 75 °C for 15 min, (7) 80 °C for 10 min, (8) marker.

result of covalently linked disulfide 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 (Figure 2). From

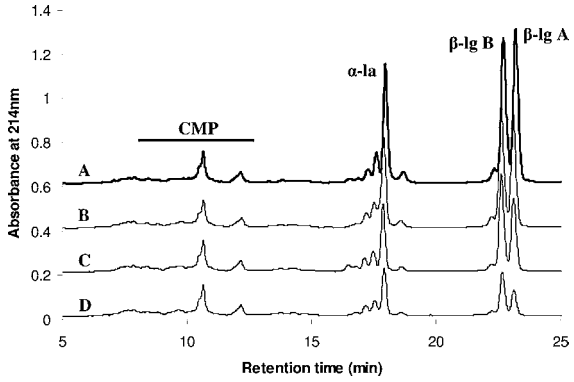

Figure 2. Reversed-phase chromatography of WPI (100 g L^{-1} protein, pH 6.4) solutions subjected to heat treatments: (A) WPI unheated control, (B) WPI 75 °C for 5 min, (C) WPI 75 °C for 15 min, and (D) WPI 80 °C for 10 min. Eluate was measured at 214 nm.

Figure 2 the greatest loss in native proteins is observed after a heat treatment of 80 °C for 10 min (D). The quantification of the protein peaks (Figure 2) from standard curves of the major whey proteins allowed for 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) to be determined. This loss in the native proteins with increasing temperature and time treatment is shown in Figure 3. CMP is a major component in WPI

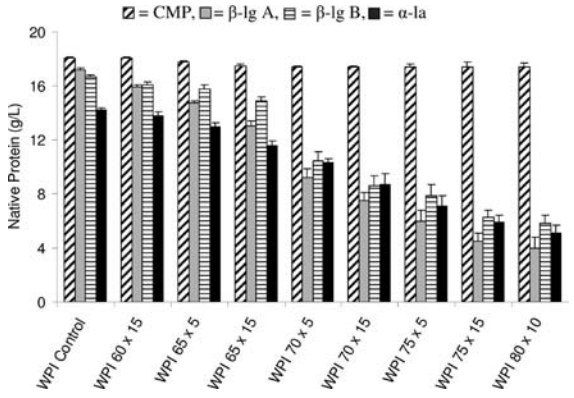

Figure 3. Native protein composition of 100 g L^{-1} untreated control and heat-treated WPI solutions subjected to different temperature (°C) × time (min) treatments determined by reversed phase-HPLC (sample mean ± SD, n = 3).

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 °C × 10 min in comparison to the unheated control. This heat resistance has been attributed to the minimal structural features of CMP which is reported to exist as an essentially disorganized macropeptide.[31]

The concentration of native β-lg A declined from 17.2 ± 0.2 g L^{-1} in control unheated WPI to 4.0 ± 0.5 g L^{-1} on heat treatment at 80 °C × 10 min. The differences in β-lg A concentration over the range of heat treatments and also with respect to the control unheated solutions was significant (P < 0.001). Figure 3 also shows a loss in native β-lg B from 16.7 ± 0.1 g L^{-1} in control untreated to 5.8 ± 0.4 g L^{-1} after 80 °C × 10 min treatment. The observed greater heat lability of β-lg A compared to β-lg B (Figure 2 and Figure 3) is in agreement with previous work.[32] 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 °C × 15 min and 70 °C × 5 min.

Heat denaturation of the proteins in the WPI solutions resulted in the loss of native proteins and extensive aggregation. SEC was utilized to distinguish the formation of aggregates. The aggregates produced on heat treatment (Figure 4 inset, Figure 5B,C) were greater than the exclusion limit of the guard column (~100 nm) or were retained during sample filtration and as a result no discernible aggregates are observed by SEC (Figure 4). 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 °C × 15 min and WPI 80 °C × 10 min heat treatments significantly reduced ($P < 0.01$) CMP concentration compared to the unheated control. During SEC elution in 20 mM sodium phosphate (pH 7) CMP exists in a multimeric form having a molecular mass between 40 and 50 kDa (Figure 4). Hydrophobically complexed multimeric forms of CMP above pH 4.5 have been reported, and this property has been exploited during CMP enrichment.[33] Overall, the results from both RP-HPLC and SEC analysis indicate that CMP, with regard 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.

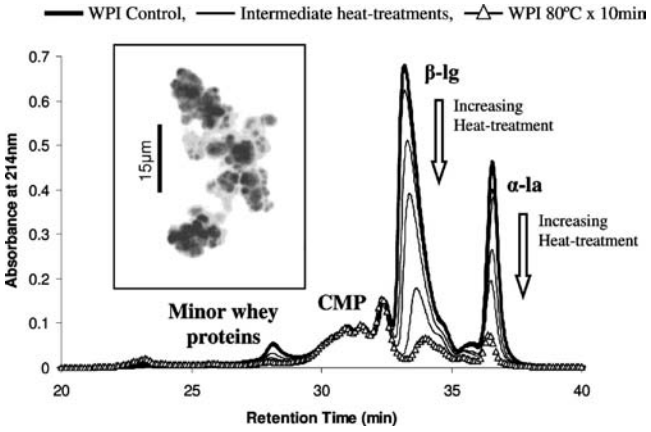

Figure 4. Size exclusion chromatography of WPI (100 g L^{-1} protein) solutions subjected to heat treatments. Samples were eluted with 20 mM Na-phosphate buffer (pH 7). Inset is a confocal laser scanning microscopy image of an isolated WPI aggregate formed after heat treatment (75 °C for 5 min).

Physicochemical Characteristics of Heat-Treated Samples. The relationships between particle size (by D. v 09, D. v 05, D. v 01, and light microscopy), turbidity (OD_{550}), solubility, viscosity, and surface hydrophobicity were compared as a function of heat treatment (see Table 1). The largest single increase in particle size (by D. v 09) occurred between 65 °C × 15 min (16.6 ± 0.2 μm) and 70 °C × 5 min (30.9 ± 0.2 μm). This also coincided with the largest single decrease in overall native protein concentration (Figure 3 and Table 1). The D. v 09 particle size increased from 15.9 ± 0.4 μm in the control unheated WPI solutions to 40.2 ± 0.6 μm in the 80 °C × 10 min WPI solutions. The D. v 05 and D. v 01 values followed a similar trend with the largest single increase occurring after a heat treatment of 65 °C × 15 min (Table 1), although heat treatments to 70 °C × 5 min are not statistically different from the unheated control ($P < 0.05$).

In this study visualization 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 °C × 15 min (17.5 ± 2.3 μm) and 70 °C × 5 min (32.9 ± 2.0 μm) heat treatments of WPI. Figure 5A–C shows the increase in the extent of aggregation from unheated control (Figure 5A) to 80 °C × 10 min (Figure 5C). Heat treatment above 65 °C × 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 °C × 10 min (Table 1). Heat-induced unfolding and subsequent aggregation led to insolubilization, resulting in a reduction in solubility from

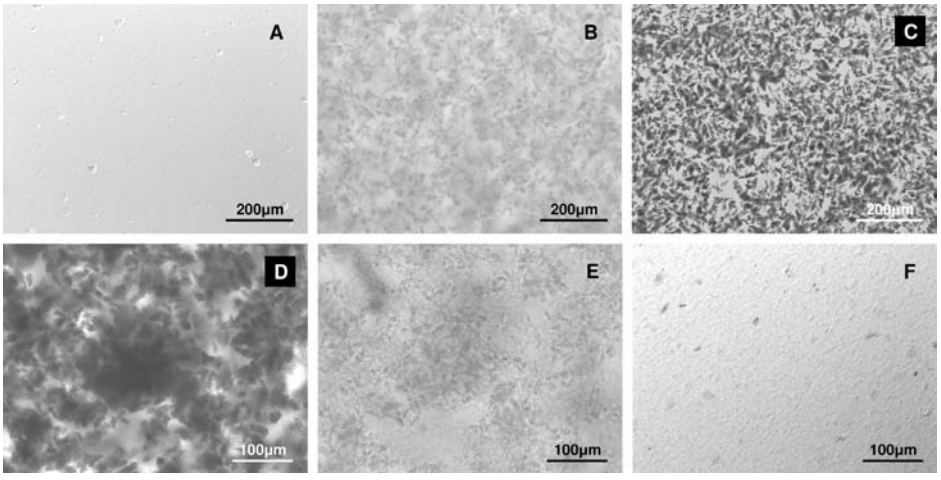

Figure 5. Light microscope images of whey protein isolate (WPI) solutions (100 g L^{-1} protein): (A) unheated control, (B) heat-treated 70 °C for 15 min, and (C) 80 °C for 10 min. Solution of 80 °C for 10 min treated WPI subsequently hydrolyzed with Corolase PP (1:100 E:S) pH 8 at (D) 2 min (0.4% DH), (E) 50 min (4.7% DH), and (F) 130 min (8.1% DH).

Table 1. Physicochemical Characteristics of Whey Protein Isolate (100 g L^{-1} Protein, pH 6.4) Solutions Subjected to Varying Temp (°C) × Time (min) Heat Treatments[a]

	particle size (μm)							
test sample	D. v 09	D. v 05	D. v 01	LM	turbidity OD_{550}[b]	solubility (%)	app viscosity (mPa s^{-1})	hydrophobicity[c]
WPI control	15.9 ± 0.4 a	5.3 ± 0.3 a	0.5 ± 0.3 a	ND[d]	0.04 ± 0.01 a	90 ± 1 a	15.5 ± 0.4 a	15.53 ± 1.27 a
WPI 60 °C × 15 min	16.4 ± 0.3 ab	5.5 ± 0.4 a	0.8 ± 0.2 a	16.2 ± 1.8 a	0.11 ± 0.02 b	84 ± 1 b	15.6 ± 0.8 ab	17.17 ± 1.50 ab
WPI 65 °C × 5 min	16.3 ± 0.5 ab	5.7 ± 0.2 a	0.6 ± 0.4 a	17.4 ± 1.6 a	0.17 ± 0.02 c	85 ± 1 b	15.6 ± 0.9 ab	20.37 ± 1.94 bc
WPI 65 °C × 15 min	16.6 ± 0.2 b	5.6 ± 0.3 a	0.7 ± 0.2 a	17.5 ± 2.3 a	0.29 ± 0.07 d	80 ± 2 c	16.1 ± 0.9 ab	23.53 ± 1.45 c
WPI 70 °C × 5 min	30.9 ± 0.2 c	10.9 ± 0.1 b	1.4 ± 0.3 b	32.9 ± 2.0 b	0.41 ± 0.05 e	77 ± 2 c	16.9 ± 0.8 b	36.71 ± 2.40 d
WPI 70 °C × 15 min	33.3 ± 0.1 d	11.2 ± 0.4 b	1.4 ± 0.4 b	35.0 ± 1.8 bc	0.65 ± 0.09 f	71 ± 2 d	20.4 ± 0.9 c	51.97 ± 3.30 e
WPI 75 °C × 5 min	36.8 ± 0.4 e	13.3 ± 0.1 c	1.6 ± 0.2 b	38.6 ± 2.5 c	0.95 ± 0.13 g	56 ± 2 e	118.4 ± 3.1 d	65.15 ± 1.91 f
WPI 75 °C × 15 min	38.9 ± 0.7 f	13.8 ± 0.5 c	1.9 ± 0.1 bc	44.6 ± 3.2 d	1.86 ± 0.20 h	43 ± 3 f	214.9 ± 6.7 e	73.69 ± 3.03 g
WPI 80 °C × 10 min	40.2 ± 0.6 g	14.8 ± 0.2 d	2.2 ± 0.3 c	45.7 ± 4.7 d	>2 i	31 ± 2 g	247.1 ± 5.0 f	70.11 ± 2.01 g

[a]Same letters within a column, a, b, c, ..., indicate no significant differences ($P < 0.05$). [b]Optical density at 550 nm (OD_{550}), protein solutions diluted to 2.5 g L^{-1} for absorbance ≤1. [c]Expressed as μg of SDS bound per 500 μg of protein. [d]ND = not determined.

90 ± 1% in the control unheated solution to 31 ± 2% solubility on heating at 80 °C × 10. The largest single decrease in solubility occurred for WPI heated at 70 °C × 15 min (71 ± 2%) and 75 °C × 5 min (56 ± 2%). The high extent of insolubilization at heat treatments greater than 70 °C 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 unheated control to 247.1 ± 5.0 mPa s^{-1} for the 80 °C × 10 min heated samples. The largest single increase in viscosity was observed to occur on heating between 70 °C × 15 min (20.4 ± 0.9 mPa s^{-1}) and 75 °C × 5 min (118.4 ± 3.1 mPa s^{-1}).

The surface hydrophobicity (SH) of the protein solutions after dialysis was expressed as μg of SDS bound per 500 μg of protein (Table 1). The disruption of hydrophobic interactions through binding of SDS facilitates the movement of the cationic methylene blue dye into the chloroform phase.[34] The SH of WPI increased by a factor of 4.75 on heating at 75 °C × 15 min (see Table 1). Interestingly, the SH decreased slightly to 70.11 ± 2.01 μg of SDS/500 μg of protein after 80 °C × 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[3,35] to the SDS complex. A previous study demonstrated that during extensive aggregation SH may be expected to decrease if aggregates were linked via noncovalent hydrophobic interactions.[36] However, the native-PAGE and SDS−PAGE results herein showed that a substantial degree of the aggregated material appeared to be linked covalently via disulfide 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 unheated 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.[37] The large aggregates, up to 40.2 ± 0.6 μm (D. v 09), were morphologically irregular when visualized by light microscopy (Figure 5C).

Many contributing factors may hasten the onset of protein aggregation. According to the three stage denaturation model,[38] 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 unheated control may have influenced the formation of larger aggregates (>30 μm) on heating at temperatures greater than 65 °C. 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[39] may induce electrostatic screening and accelerate the aggregation process.[5,40,41] In addition, the use of a relatively high protein concentration (100 g L^{-1} protein) herein may also have promoted aggregation.[4,42]

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 (Figure 6). Previous studies have demonstrated improved reaction rates as a consequence of pretreatment of whey protein substrates.[12,43]

The TNBS assay for DH determination gave higher results for the end-point DH than the pH-stat methodology (Table 2), which is in agreement with a previous study.[44] However, none of the end-point DH values determined by TNBS were significantly different ($P < 0.05$) from each other. The time to

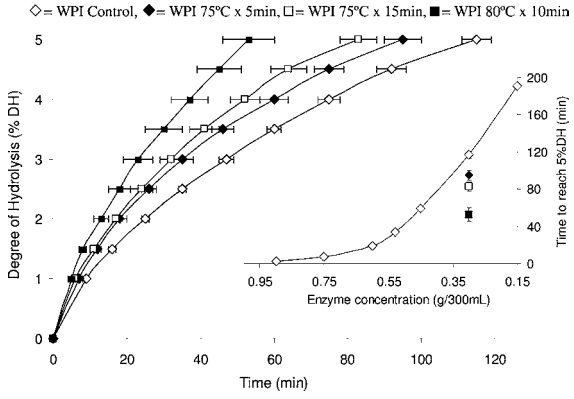

Figure 6. Hydrolysis profiles for whey protein isolate (100 g L^{-1} protein, pH 8) unheated control and heat-treated solutions to a degree of hydrolysis (DH) of 5% with Corolase PP (1:100 E:S) as obtained using the pH-stat method. Inset is of 100 g L^{-1} protein WPI control solutions which were subjected to hydrolysis with varying concentrations of enzyme, and the time to reach a DH of 5% was measured.

Table 2. Physicochemical Characteristics of Whey Protein Isolate (100 g L^{-1} Protein) and Hydrolyzed, to a Degree of Hydrolysis of 5% with Corolase PP (1:100 E:S), Unheated Control, and Heat-Treated Solutions

test sample	deg of hydrolysis (%)[a]	particle size (μm)				turbidity OD_{550}[b]	solubility (%)	app viscosity (mPa s^{-1})	hydrophobicity[c]
		D. v 09	D. v 05	D. v 01	LM				
WPI control	ND	15.9 ± 0.4 a	5.3 ± 0.3 a	0.5 ± 0.3 a	ND[d]	0.04 ± 0.01 a	90 ± 1 a	(15.5 ± 0.4a)[e] 16.1 ± 0.2 a	15.53 ± 1.27 a
hydrolysates									
WPI control	6.54 ± 0.23 a	13.5 ± 0.5 b	4.8 ± 0.3 a	0.7 ± 0.2 a	ND	0.03 ± 0.01 a	92 ± 2 a	13.9 ± 5.1 a	17.49 ± 2.31 a
WPI 75 °C × 5 min	6.91 ± 0.30 a	20.3 ± 0.6 c	6.7 ± 0.4 b	1.0 ± 0.4 ab	21.4 ± 2.8 a	0.83 ± 0.08 b	59 ± 2 b	97.2 ± 3.5 b	73.06 ± 2.63 b
WPI 75 °C × 15 min	6.52 ± 0.36 a	20.2 ± 0.4 c	7.0 ± 0.2 b	1.1 ± 0.5 ab	25.2 ± 4.2 a	0.97 ± 0.25 bc	50 ± 3 c	105.8 ± 7.9 bc	81.72 ± 1.66 c
WPI 80 °C × 10 min	6.78 ± 0.27 a	21.8 ± 0.5 d	7.1 ± 0.3 b	1.2 ± 0.3 b	23.3 ± 7.3 a	1.26 ± 0.24 c	42 ± 3 d	113.1 ± 7.4 c	83.97 ± 2.59 c

[a]Degree of hydrolysis (DH) determined by TNBS; a peptide bond nitrogen content value of 123.3 mg g^{-1} was used. [b]Optical density at 550 nm (OD_{550}), protein solutions diluted to 2.5 g L^{-1} for absorbance ≤1. [c]Expressed as μg of SDS bound per 500 μg of protein. [d]ND = not determined. [e]Apparent viscosity determined at pH 6.4.

reach a DH of 5% (6.78 ± 0.27% by TNBS) for a WPI solution subjected to prehydrolysis heat treatment of 80 °C × 10 min (53 ± 7 min, 6.78 ± 0.27% by TNBS) was approximately half that of an unheated control WPI solution (115 ± 4 min, 6.54 ± 0.23% by TNBS). Heating WPI solutions (100 g L^{-1}) to 80 °C for 10 min followed by hydrolysis, at an E:S of 1:100, gave an equivalent reaction time to unheated control WPI solutions (100 g L^{-1}) hydrolyzed at an E:S of 1.5:100 (Figure 6 inset). Solutions which were subjected to prehydrolysis treatment at temperatures ≥75 °C × 5 min had a statistically significant increased rate of hydrolysis ($P < 0.001$) compared to the unheated control. A 72% loss in native protein concentration in WPI solutions heated at 80 °C for 10 min compared to the unheated control resulted in a 54% decrease in hydrolysis time to achieve a DH of 5% (Figure 6). Those samples subjected to heat treatments 60 °C × 15 min, 65 °C × 5 min, 65 °C × 15 min, 70 °C × 5 min, and 70 °C × 15 min displayed similar hydrolysis curves to control untreated WPI and were not statistically different from the control hydrolysis reaction using the comparative Tukey test ($P < 0.05$). Therefore, the polymerization of the whey proteins into aggregates possessing a D. v 09 greater than or equal to 36.9 μm (Table 1) corresponding to a total loss of native protein greater than or equal to 41% (Figure 3) presented a conformation which was beneficial to enzymatic hydrolysis with Corolase PP (Figure 6).

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 internalize and protect certain residues from hydrolytic cleavage.[45] 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[46] where large aggregates possess a much lower diffusion coefficient than the enzyme. However, this was minimized for our hydrolysis experiments which were performed under moderate stirring.

Physicochemical Characteristics of Hydrolysates. Table 2 summarizes the physicochemical characteristics of the non-heat-treated control, and the treatments showing the greatest increase in reaction rate (namely, WPI 75 °C × 5 min, 75 °C × 15 min, and 80 °C × 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 °C × 10 min (Table 2) compared to the unhydrolyzed 80 °C × 10 min solution (Table 1). Table 2 also shows that hydrolysis of the most extensively heat-denatured WPI solutions, namely, 75 °C × 5 min, 75 °C × 15 min, and 80 °C × 10 min, resulted in an increase in solubility of the solution at 5% DH compared to the respective unhydrolyzed samples (Table 1). The increase in solubility was greatest in the WPI 80 °C × 10 min hydrolysates, which showed an increase in solubility of 9 ± 4% compared to the unhydrolyzed WPI 80 °C × 10 min heat-treated substrate prior to hydrolysis. The heat-inactivation step (85 °C for 20 min) for the enzyme is a further heat treatment which influences the physicochemical properties of the solutions. Samples in which the enzyme was inactivated by lowering the pH to 2.5 with 2 M HCl showed altered

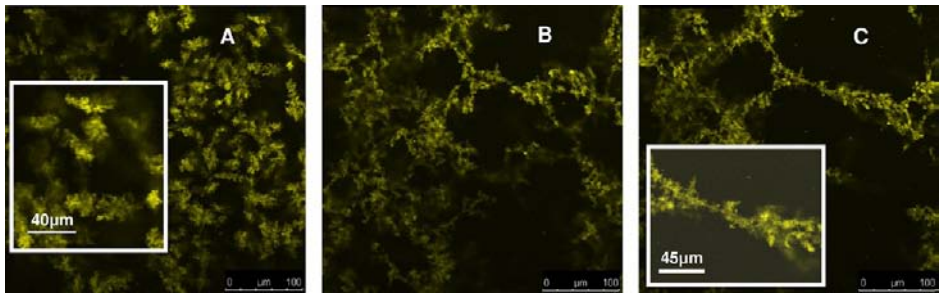

Figure 7. Confocal laser scanning microscopy (CLSM) images of whey protein isolate (100 g L^{-1} protein) subjected to heating at 80 °C for 10 min, stained with acridine orange, and subsequently hydrolyzed with Corolase PP (1:100 E:S) at (A) 5 min (0.9% DH), (B) 30 min (3.4% DH), and (C) 56 min (5% DH).

physicochemical characteristics from the heat-inactivated hydrolysates (results not shown).

The changes in apparent viscosity taking place during hydrolysis of unheated control and heat-treated solutions of WPI samples were monitored through rotational rheological analysis. Over the course of the hydrolysis reaction the mean apparent viscosity of the WPI non-heat-treated control solution decreased: from $1.61 \pm 0.69 \times 10^{-2}$ Pa/s at 0.2% DH to $1.39 \pm 0.51 \times 10^{-2}$ Pa/s at 5% DH. On the other hand, the apparent viscosity of the WPI 75 °C × 5 min, 75 °C × 15 min, and 80 °C × 10 min solutions decreased significantly ($P < 0.001$) at the 5% DH level (Table 2) compared to their unhydrolyzed equivalents (Table 1).

SH of the hydrolysates (Table 2) showed an increase in comparison to the respective unhydrolyzed starting solutions (Table 1). The greatest mean percentage increase in SH was 16.5% in the 80 °C × 10 min hydrolysate (83.97 ± 2.59 μg of SDS/500 μg of protein) compared to the unhydrolyzed 80 °C × 10 min solution (70.11 ± 2.01 μg of SDS/500 μg of protein). This trend is in agreement with previous work on a heat-denatured WPI.[47] However, peptide−protein hydrophobic interactions might have influenced the determinations.

Microscopic Analysis during the Course of Hydrolysis. Visualization of the structural changes occurring in the WPI as a result of enzymatic hydrolysis was achieved through microscopic analysis. In the solutions which were heat-treated prior to enzymatic hydrolysis, it was possible to discern the destabilization of aggregates by LM. Figure 5D−F shows the changes in WPI heat treated at 80 °C × 10 min during subsequent hydrolysis. Figure 5E shows the solution at 4.7% DH (t_{+50} min), where a noticeable reduction in the extent of aggregation is observed compared to Figure 5D (t_{+2} min). Allowing the enzymatic reaction to continue to 8.1% DH (Figure 5F) 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}, results not shown) at 8.1% DH of the 80 °C × 10 min heat-treated solution was similar to the turbidity (0.17 ± 0.02, OD_{550}) of a 65 °C × 5 min heat-treated solution prehydrolysis (Table 1).

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[48] utilized microscopy (TEM) to visualize 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 (Figure 7) the formation of morphologically irregular aggregates is apparent in the hydrolyzed WPI subjected to preheating at 80 °C for 10 min. From Figure 7A the aggregates appeared to be quite regularly distributed after 5 min of hydrolysis. However, large gaps began to appear as hydrolysis proceeded, and an aggregate network began to form (Figure 7B,C) as aggregate size (by D. v 09) 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).

Aggregation of WPI by thermal pretreatment 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 °C 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. Utilizing various analytical techniques allowed for the quantification and visualization 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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Funding

The work herein was funded by Enterprise Ireland as part of the Food for Health Ireland project, Grant No. CC20080001. I.B.O'L. was funded by Enterprise Ireland under the Teagasc Walsh Fellowship Scheme.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors wish to acknowledge the help and assistance gratefully provided by the staff of the National Food Imaging Centre, Moorepark, Fermoy, Ireland, especially M. A. Auty.

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