



The effect of gel structure on the kinetics of simulated gastrointestinal digestion of bovine β -lactoglobulin

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ABSTRACT

The structure and properties of protein gels depend on the conditions under which they are formed. Here, we assessed the susceptibility of protein to simulated gastro-duodenal digestion of weak gels with contrasting structures, produced from either purified bovine β -lactoglobulin (β -Lg) or whey protein isolate (WPI) at pH ranging from 2.5 to 6.5 and using different heating regimes. Gels formed close to the isoelectric point proved to be very resistant to simulated gastric digestion, with more than 85% of β -Lg remaining and in the simulated duodenal phase of digestion. The sample heated to 85 °C was most resistant with over 40% remaining. In the WPI sample heated to 85 °C, more than 20% of the original β -Lg content remained undigested after simulated gastro-duodenal proteolysis. These results suggest that firm particulate gels can persist longer in the GI tract and may be useful in inducing satiety and thus provide another weapon in the fight against obesity.

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1. Introduction

Protein gels are widely used to provide structure in food systems and whey proteins have been shown to form a wide range of gel structures depending on the conditions used (Nicolai, Britten, & Schmitt, 2011; van Vliet, Lakemond, & Visschers, 2004). The main protein (60–70%) in whey is β -lactoglobulin (β -Lg), which is a globular protein from the lipocalin structural family. This protein consists of 162 amino acids organised primarily into a beta structure with a hydrophobic calyx. The protein has complex equilibrium aggregation behaviour depending on environment (Sawyer & Kontopidis, 2000) and is normally found as a dimer under physiological conditions. β -Lg exists as a dimer at high and neutral pH, forming octomers at pH values between 4 and 6 before dissociating to give a mixture of monomers and dimers at pH values below 3 (Uhrinova, Smith, Jameson, Uhrin, Sawyer, & Barlow, 2000).

β -Lg readily unfolds above its main transition temperature of 75 °C and the associated exposure of hydrophobic amino acid residues leads to aggregation, especially close to the isoelectric point where the net charge is low. In addition, an important factor in the aggregation behaviour of β -Lg has been shown to be the free thiol of Cys121, which provides the potential of intermolecular disulphide bridging (Alting, Hamer, de Kruif, Paques, & Visschers,

2003). The heating of β -Lg causes the formation of aggregates, which then join together to form gels with different structures, such as fine stranded or particulate, depending on pH (Langton & Hermansson, 1992). The rheological properties of these gels have also been shown to be very different and pepsin hydrolysis at pH 3.35 prior to heating to 85 °C has been shown to increase aggregation and thus viscosity (Mudgal, Daubert, Clare, & Foegeding, 2011).

The resistance of β -Lg to digestion, particularly in the gastric compartment, coupled with its absence from human milk has been proposed to contribute to the allergenicity of bovine β -Lg in humans (Wal, 2004). In addition to the intrinsic susceptibility of a given scaffold to proteolysis, the three-dimensional structure of a protein may facilitate interactions with other components found in foods or the gastrointestinal (GI) tract lumen, thus altering their stability to proteolysis. Indeed, polysaccharides, including pectins, gum arabic and xylan, may retard the susceptibility of β -Lg to degradation by trypsin and chymotrypsin (Mouecoucou, Villaume, Sanchez, & Mejean, 2004). We have previously shown that phosphatidylcholine (PC), a phospholipid abundant in milk and also secreted by the gastric mucosa, interacts with the milk protein α -lactalbumin slowing its breakdown during *in vitro* simulated gastric digestion (Moreno, Mackie, & Mills, 2005). We have also reported the effects of physiological surfactants on the simulated GI proteolysis of bovine β -Lg, showing that under certain conditions β -Lg is almost completely protected from simulated digestion (Mandalari, Mackie, Rigby, Wickham, & Mills, 2009). Similar work

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using *in vitro* digestion models has shown that food processing including emulsification can alter rates of protein digestion and the patterns of peptides produced (Dupont et al., 2010; Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009). These articles show the potential for using processing and food structuring to alter the production and persistence of bioactive peptides or allergenic epitopes.

Whilst there have been few studies on the digestion of gelled protein, there has been some recent work on the digestion of β -Lg cross-linked with laccase (Tantoush et al., 2011), as well as digestion of fibrils formed from β -Lg (Bateman, Ye, & Singh, 2010). The cross-linked protein was shown to be more resistant to proteolysis in contrast to the β -Lg fibrils, which were digested by pepsin in a few minutes. Similar improvements in resistance to proteolysis as a result of cross-linking have also been shown by others using a range of enzymes and substrates (Giosafatto, Rigby, Wellner, Ridout, Husband, & Mackie, 2012; Mariniello, Giosafatto, Di, Sorrentino, & Porta, 2007; Tang, Li, & Yang, 2006). In a study looking at the release of iron from whey protein hydrogels (Remondetto, Beyssac, & Subirade, 2004), the authors concluded that different iron release profiles could be obtained depending on the microstructure of the gels in which the iron was trapped. Fine stranded gels, prepared in the presence of 10 mM Fe^{2+} , differed from particulate gels, prepared in the presence of 30 mM Fe^{2+} . The results showed that more iron was released from fine stranded gels than from particulate gels, indicating that the rate of digestion of the gel was dependant of the type of gel structure; fine strands or coarse particles.

In this work we have studied the impact of a range of gel structures on the resistance of a single protein (β -Lg) to simulated upper GI tract digestion. The weak gels were formed using a range of thermal processes at different pH values and the products of *in vitro* digestion assessed by quantitative analysis of SDS–PAGE.

2. Materials and methods

2.1. Protein heat-treatment

Purified β -lactoglobulin (90% β -Lg) from bovine milk was purchased from Sigma–Aldrich (L3908; Poole, UK), and whey protein isolate (WPI; BiPRO®, 93% protein) supplied by Davisco Foods International Inc. (Eden Prairie, MN). β -Lg samples were made up at 40 g/l in 0.15 M NaCl (final pH 6.5, 5.2, 4.8 or 2.5) and heated to 70 °C for 24 h (0.5-ml aliquots in 1.5-ml sealed plastic tubes), which is not a commercially relevant process, therefore we also heated β -Lg and WPI samples (40 g/l, pH 5.2) to 85 °C for 0.5 h. It is clear that such low protein concentrations form weak gels. These proved much more amenable to analysis postdigestion than firm gels, which tend to retain the digested peptides and are thus difficult to analyse reproducibly. The amount of the protein incorporated into the gel matrix was determined by centrifuging the samples at 13,000 rpm (11,600g) for 10 min. The amount of soluble protein remaining in the clear supernatant formed above the separated gelled phase was assayed. The supernatant was carefully removed using a syringe and needle, passed through a 0.22 μm filter, and the protein concentration determined by the OPA method (Church, Swaisgood, Porter, & Catignani, 1983). The proportion of gelled protein, given in Table 1, was calculated from the measured amount of soluble protein remaining in solution after heating.

2.2. Fourier transform infra-red spectroscopy (FTIR)

FTIR spectra were measured for unheated β -Lg samples (40 g/l in 0.15 mM NaCl at various pH) and for the heat-treated samples, after separating the gelled phase of a sample from the aqueous phase containing the remaining soluble protein, as described above. Both, the gelled and the soluble phases were analysed.

Spectra were collected in attenuated total reflection mode (FTIR-ATR) in duplicate using a Nicolet 860 FTIR spectrometer (Thermo Nicolet, Hemel Hempstead, UK) equipped with a GoldenGate single reflection diamond ATR sampling accessory (Specac Ltd., Orpington, Kent, UK). For each spectrum 256 scans at 2 cm^{-1} resolution were averaged, using automatic vapour suppression and the empty crystal as reference. The water spectrum was subtracted manually. To enhance the resolution of the amide bands, Fourier deconvolution using 25 cm^{-1} bandwidth and enhancement factor of 1.5 was used with the spectrometer software (Omnic 7).

2.3. Scanning electron microscopy (SEM) of protein gels

Protein gels were fixed with 3% glutaraldehyde (Agar Scientific, Stansted, UK) in 0.1 M cacodylate buffer (pH 7.2) overnight. This was followed by 3×15 min washes with 0.1 M cacodylate buffer and dehydration through a series of ethanol solutions (10, 20, 30, 40, 50, 60, 70, 80, 90, $3 \times 100\%$) for at least 20 min in each. The gel was critical point dried in a Polaron E3000 critical point drier (Quorum Technologies, Newhaven, UK) using liquid carbon dioxide as the transition fluid. The pieces of gel were split with a razor blade and allowed to tear apart to reveal a free-break surface. The pieces were mounted on aluminium SEM stubs using sticky tabs with the torn surfaces facing upwards. The samples were coated with gold in an Agar high resolution sputter-coater apparatus. SEM was carried out using a Zeiss Supra 55 VP FEG SEM (Carl Zeiss, Germany), operating at 3 kV.

2.4. ζ -Potential measurements

The gel structures produced after heat-treatment were broken by sonication using a probe sonicator (Digital Sonifier 250, Branson Ultrasonics Corp., CT) working at 20% amplitude (3×1.5 s pulses), prior to ζ -potential measurement. The ζ -potential was measured using a Nano-ZS Zetasizer (Malvern Instruments Ltd., Malvern, UK). Prior to analysis, the sonicated samples were diluted about 400 times with 0.15 M NaCl (at the same pH as the sample being analysed). Diluted samples were then injected into a DTS1060 folded capillary cell (Malvern Instruments Ltd.). Each sample was analysed at least 20 times and the results displayed as a mean. Data are shown as the average values obtained from three samples prepared under the same heat-treatment conditions.

2.5. Rheology

Protein gelation was monitored *in situ* by dynamic oscillatory rheometry using a controlled strain AR2000 rheometer (TA Instruments, UK). The protein solution (13 ml, 40 g/l in 0.15 M NaCl, at various pH) was introduced into the measurement cell of the rheometer using the coaxial cylinder geometry (15 mm o.d., 12 mm i.d.). A thin layer of low-viscosity silicone oil was spread over the surface of the protein solution to prevent evaporation. Oscillation tests were performed at 70 or 85 °C at a single frequency (1 Hz) to study the development of viscoelasticity over time (evolution of the storage modulus G') monitored at a shear strain of 1% for up to 24 h. Only the samples that did not phase separate during heating were analysed.

2.6. Preparation of phospholipid vesicles

PC vesicles were used in the *in vitro* model of digestion to mimic phospholipid vesicles secreted by the gastric mucosa. Their preparation has been described in detail elsewhere (Macierzanka et al., 2009, 2011c). In brief, the solvent was evaporated from a 0.94 ml aliquot of egg L- α -phosphatidylcholine (PC, 99% purity; Lipid Products, South Nutfield, UK) stock solution (50 mg/ml chloroform).

Table 1

Characteristics of the heat treated protein samples.

Protein sample	Heat treatment	Gelled protein (% of total protein) ^c	Monomeric β -Lg after the heat treatment (% of initial β -Lg) ^{a,c}
β -Lg	pH 6.5, 70 °C, 24 h	89.7 $\pm$ 1.4	65.6 $\pm$ 6.9
β -Lg	pH 5.2, 70 °C, 24 h	50.6 $\pm$ 3.1	92.5 $\pm$ 6.3
β -Lg	pH 4.8, 70 °C, 24 h	51.9 $\pm$ 3.0	91.5 $\pm$ 2.5
β -Lg	pH 2.5, 70 °C, 24 h	71.0 $\pm$ 3.3	53.1 $\pm$ 5.3
β -Lg	pH 5.2, 85 °C, 0.5 h	92.6 $\pm$ 1.0	97.5 $\pm$ 2.8
WPI (total heat-treated) ^b	pH 5.2, 85 °C, 0.5 h	91.4 $\pm$ 0.2	85.4 $\pm$ 4.0
WPI (gelled phase) ^b	pH 5.2, 85 °C, 0.5 h	100	100.2 $\pm$ 4.4
WPI (serum phase) ^b	pH 5.2, 85 °C, 0.5 h	0	52.9 $\pm$ 3.5

^a Determined by SDS–PAGE (lane labelled C2) under reducing conditions.^b The gelled and the serum phases were obtained after centrifugation of the heated WPI samples (i.e. WPI total heat-treated samples) as explained in the Section 2.^c The results are mean $\pm$ standard deviation.

The thin film of phospholipids formed was then suspended in 6.1 ml of 0.15 M NaCl, pH 2.5 (37 °C, 170 rpm for 30 min). The suspension was transferred to an ice-jacketed vessel and repeatedly sonicated until it was clear. The dispersion was prepared on the day of digestion experiment and incubated at 37 °C for 20 min prior to use.

2.7. *In vitro* simulated gastro-duodenal digestion

The *in vitro* proteolysis experiments were performed in triplicate for samples obtained from each of the heat-treatment conditions studied. The samples were cooled to room temperature and digested as described below. The digestion model is based on the procedure described previously (Macierzanka et al., 2009, 2011b) and is representative of physiologically relevant conditions. During the experiments samples were collected periodically and analysed using SDS–PAGE. Control experiments were also undertaken for unheated β -Lg samples. Heated WPI samples were used either directly after the heat-treatment (such samples have been referred as to the total heat-treated samples) or after additional separation of the samples into a gelled phase and a soluble (serum) phase, following the heat treatment. The separation was achieved by centrifugation at 13,000 rpm (11,600g) for 10 min. The proteins in the two phases were then digested separately using the given digestion method in order to provide comparative data regarding the digestibility of different structural components of the same sample (i.e. gelled vs. soluble protein after the heat-treatment). All digestion experiments used constant protein:enzyme ratios as described further in the text.

2.8. *In vitro* simulated gastric digestion

Following heat-treatment of the protein samples (0.5 ml aliquots in individual 1.5 ml tubes), 0.3 ml of vesicular PC was added to each sample, the pH of the mixture adjusted to 2.5 and the volume made up to 0.9 ml with 0.15 M NaCl, pH 2.5. After 10-min incubation at 37 °C, 100 μ l of a solution of porcine pepsin (P6887, Sigma, Poole, UK; activity: 3300 U/mg of protein calculated using haemoglobin as substrate) dissolved in 0.15 M NaCl (pH 2.5) at the concentration of 10 g/l were added. The final concentration of the protein (β -Lg or WPI) in the digestion mix was 20 g/l, the PC concentration was 2.32 g/l and the protein:pepsin ratio was 1:20 w/w. The samples were digested under continuous moderate agitation (170 rpm) at 37 °C for up to 60 min. Proteolysis was stopped after 0.25, 1, 2, 5, 10, 30 and 60 min (gastric time-point samples) by mixing with 200 ml of 0.5 M ammonium bicarbonate to immediately raise the pH over 7.5 and irreversibly inactivate pepsin. Seven additional samples of protein gel were prepared in the same way and digested for 60 min with pepsin. Proteolysis in those seven samples was terminated by mixing with 260 μ l 0.1 M NaOH, increasing the pH to 7.5. The samples were kept at 37 °C for 15 min before being transferred to the simulated duodenal compartment of digestion.

2.9. *In vitro* simulated duodenal digestion

The pH of the samples for duodenal digestion was adjusted to 6.5 with 0.1 M HCl/NaOH and the digestion was followed for up to 30 min at 37 °C (170 rpm), mimicking transfer of the gastric digesta into duodenal conditions and its subsequent degradation. Each sample was made up with a 0.5 M Bis-Tris buffer (pH 6.5; Sigma, Poole, UK) to a final Bis-Tris concentration of 25 mM. Additionally, a mixture of bile salts (BS; 25 μ l in 0.15 M NaCl, pH 6.5), comprising equimolar quantities of sodium taurocholate (T4009, Sigma, Poole, UK) and sodium glycodeoxycholate (G9910, Sigma, Poole, UK), was added to a final total concentration of 7.4 mM. The digestion was performed with duodenal proteases, porcine trypsin (activity: 13,800 U/mg of protein calculated using BAEE as substrate) and bovine α -chymotrypsin (activity: 40 U/mg of protein calculated using BTEE as substrate). Both enzymes were purchased from Sigma (T0303 and C7762, respectively). The enzymes were dissolved in 0.15 M NaCl, pH 6.5, (2.5 g/l and 10 g/l, respectively) and added to the digestion samples to give final enzyme:protein ratios of 1:400 (w/w) for trypsin and 1:100 (w/w) for chymotrypsin in all analysed systems. The proteolysis in individual samples was stopped after 0.25, 1, 2, 5, 10, 15 and 30 min (duodenal time-point samples) by mixing with 100 μ l solution of Bowman-Birk trypsin–chymotrypsin inhibitor (T9777 Sigma, Poole, UK) dissolved in 150 mM NaCl, pH 6.5, at a concentration calculated to inhibit twice the amount of the duodenal enzymes used.

2.10. Characterisation of digestion samples

The gel structures remaining after digestion were sampled at specific time-points and broken by sonication using a probe sonicator (Digital Sonifier 250, Branson Ultrasonics Corp., CT) working at 20% amplitude (3 $\times$ 1.5 s pulses). The procedure was required to ensure that the digestion could be followed reproducibly by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). The analysis of the digestion samples was undertaken as described in detail elsewhere (Macierzanka et al., 2009, 2011c). Briefly, all chemicals and gels used for the SDS–PAGE analysis were purchased from Invitrogen Ltd. (Paisley, UK). Samples containing 15 μ g of protein were loaded onto a 10% polyacrylamide NuPAGE Novex Bis-Tris precast gel. Gels were run for 35 min at 120 mA/gel and 200 V, and stained using a colloidal Coomassie stain (SimplyBlue SafeStain). A SeeBlue Plus2 (1 $\times$) prestained molecular weight marker comprising a mixture of proteins ranging in *M_r* from 3 to 188 kDa was used. Densitometric analyses of protein patterns were made using TotalLab TL120 software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). The analysis consisted of the identification of the lanes, subtracting the background and then identification of the position and the intensity of bands. Gaussian curves were subsequently fitted to the bands. Finally, the area under the curve corresponding to each band was

calculated (Macierzanka et al., 2009, 2011a). At least two replicate gels of digestion samples were analysed. Concentrations of β -Lg in the digestion time-point samples were calculated by fitting the protein band intensity to the standard curve (Fig. S1 in the Supplementary Information, SI) obtained for a range of β -Lg concentrations analysed by SDS–PAGE in the same manner as the digestion samples. Selected digestion samples were also analysed by SEM using the procedure described above. These samples were not sonicated before the microscopic examinations.

3. Results

When heat-set gels are formed they nucleate to form “microgels” that subsequently join together into a network. In the work described here we have used heating for long times (24 h) at 70 °C and at a range of pH values to form different gel structures from β -Lg (Fig. 1a–d). At pH 6.5 the charge on the protein is negative as the pH is above the isoelectric point (Table 2). This leads to the formation of a continuous firm white gel. Pictures of all the gels are shown in the supplementary information (Fig. S2). As the pH was decreased towards the isoelectric point the protein was no longer able to form a continuous network and an aggregated network was formed in a clear aqueous phase. This was true of the gels formed both at pH 5.2 and pH 4.8 (Fig. S2b and c). The final sample was formed at pH 2.5 where the charge on the protein was large and positive (Table 2) and resulted in a cloudy viscous liquid (Fig. S2d). The structure of these four systems was assessed by SEM and as expected showed large differences, especially in the size of the microgels. Figs. 1a and S3a show that at pH 6.5 a fine stranded gel was formed by the nucleation of many microgel particles of about 20 nm in diameter. This is in marked contrast to the samples heated at pH 5.2 and 4.8 shown in Fig. 1b and c respectively, which generated coarser systems in which the microgels were micron sized. The sample heated at pH 2.5, Figs. 1d and S3b, resembled that formed at pH 6.5 in that a fine stranded network was formed with microgels that were tens of nanometres in size. In an attempt to investigate the effect of heating conditions, we also heated samples at pH 5.2 and 85 °C for 30 min. This led to the formation of a continuous firm white gel (Fig. S2e) that was formed from microgels that were again micron sized (Fig. 1e).

The molecular structure of the gels was also investigated in more detail using FTIR. Fig. 2a shows Fourier deconvolved spectra for the amide bands of unheated β -Lg control samples at the four different pH values used. All show a main amide 1 peak at 1630 cm^{-1} , which is indicative of the intramolecular β -sheet that typifies the structure of β -Lg. After heating, there is considerable variation between the different gelled samples as seen in Fig. 2b. At pH 5.2 and 4.8 the spectra remain quite similar to the unheated samples showing a predominance of intramolecular β -sheet. However, the samples heated at pH 6.5 and 2.5 as well as the sample heated to 85 °C (pH 5.2), all show a broadening of the main amide 1 peak and a shift to lower wavenumbers. This is indicative of intermolecular β -sheet that has been induced by the aggregation process. Interestingly, the protein left in solution after heating showed very similar spectra to the unheated samples, Fig. 2c. Unfortunately, there was insufficient protein left in solution after heating the β -Lg to 70 °C at pH 6.5 or 85 °C at pH 5.2 to obtain good quality spectra.

In addition to the molecular structure of the gels, their viscoelasticity was also measured. Fig. 3 shows the elastic moduli (G') of the three β -Lg samples that formed a continuous gel. The value of G' is shown as a function of time as the gels developed. There were marked differences in the behaviour of β -Lg under the different conditions. When heated to 70 °C, the kinetics of gel formation was quite slow, taking many hours to occur. This is in contrast to

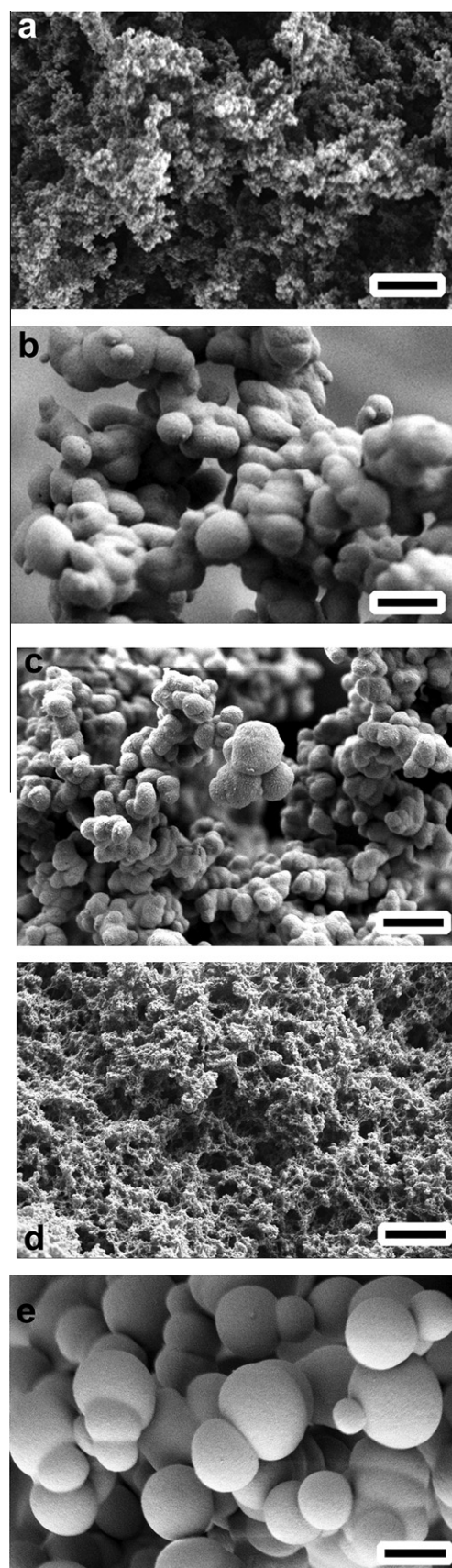


Fig. 1. SEM images of β -Lg gels heated at 70 °C for 24 h at pH 6.5 (a), pH 5.2 (b), pH 4.8 (c) and pH 2.5 (d). β -Lg gel heated at 85 °C for 30 min at pH 5.2 (e). The scale bars correspond to 2 μm .

Table 2
Characteristic parameters of the β -Lg samples.

pH	Zeta potential (mV)	Heating temperature (°C)	Size of microgel (nm)	Residual β -Lg after gastric digestion (%)	Residual β -Lg after duodenal digestion (%)
2.5	18.2 ± 0.7	70	20–50	76	8
4.8	-0.1 ± 2.8	70	500–2000	91	14
5.2	-5.1 ± 2.3	70	1000–2000	87	25
6.5	-14.1 ± 2.2	70	10–50	24	9
5.2	-6.6 ± 0.3	85	1000–3000	79	43

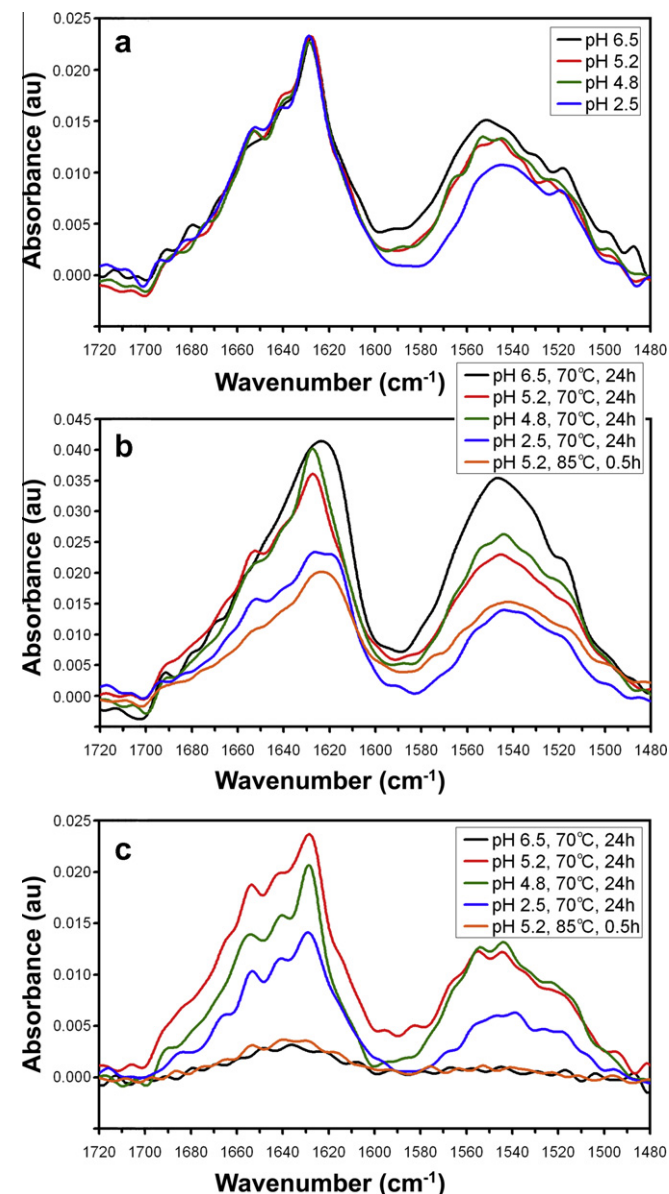


Fig. 2. FTIR (Fourier deconvolution) of the β -Lg samples; (a) unheated controls at 40 g/l protein concentration and various pH values, (b) gelled phases, separated after heating, and (c) remaining protein solutions (serum phase) separated from the gelled phases after heating (the heating conditions given in the legends (b, c)).

the gel formed at 85 °C, which formed in a few minutes. In addition, the gel formed at pH 2.5 was much less elastic than the other gels. This could well have been because of acid hydrolysis.

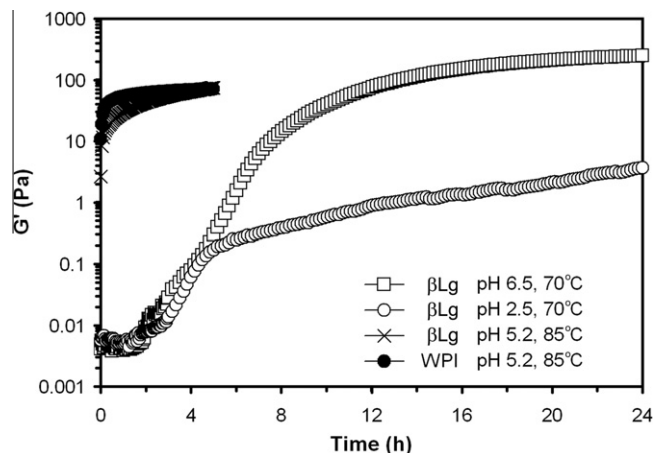


Fig. 3. Evolution of G' over the time of gelation for β -Lg or WPI samples (the heating conditions given in the legend).

Having produced samples with a range of microstructural characteristics, we put them through our standard *in vitro* digestion protocol, simulating gastric followed by duodenal digestion. During both stages of digestion, samples were periodically taken and analysed by SDS–PAGE in order to follow the progress of proteolysis. The results are shown in Fig. 4 and reveal significant differences in rates of proteolysis. Fig. 4a and d shows the analysis of digesta from samples heated at pH 6.5 and 2.5 respectively. These two samples show significant proteolysis through simulated gastric digestion, with the appearance of an increasing number of peptides of decreasing size with time. The sample heated at pH 2.5 also showed significant acid hydrolysis (Fig. 4d, control C2) during heating. The sample heated at pH 6.5 showed the limited formation of β -Lg oligomers that were resistant to the reducing conditions of the SDS–PAGE (Fig. 4a, control C2). Unheated β -Lg was also digested under the conditions used in the current study, and the results are shown in Fig. S4. Only limited pepsinolysis was observed for all other heated samples (Fig. 4b, c, e). The SDS–PAGE results for the duodenal stage of digestion show similar differences, with the sample heated at pH 2.5 being the least resistant (Fig. 4d) along with the sample heated at pH 6.5 (Fig. 4a). The samples heated to 70 °C at pH 5.2 and pH 4.8 were quite resistant to simulated duodenal digestion (Fig. 4b, c), but the most resistant was that heated to 85 °C at pH 5.2 (Fig. 4e).

In order to quantify the differences seen in the digestion of the samples, the SDS–PAGE gels were analysed by densitometry and the results are shown in Fig. 5a. This figure confirms what was seen in the gels and highlights the marked differences in the susceptibility of the samples to proteolysis. The unheated β -Lg control shows the most resistance to pepsin, but is subsequently degraded through simulated duodenal digestion. This is in marked contrast to the sample heated at pH 6.5, which quickly degraded through simulated gastric digestion, leaving just over 20% of the β -Lg intact at the end of this phase of digestion. Whilst the sample heated at pH 2.5 proved to be more resistant to pepsin, both the sample heated at pH 6.5 and 2.5 were significantly degraded through simulated duodenal digestion and only ca. 10% intact β -Lg remained at the end. There was little correlation between the resistance of the samples to simulated gastric conditions and simulated duodenal conditions. For example, the sample heated to 85 °C (pH 5.2) was the most resistant to duodenal digestion with over 40% of the β -Lg remaining intact at the end. However the same sample showed relatively poor resistance to pepsin, with only ca. 80% of the β -Lg remaining intact in comparison to over 95% for the unheated sample.

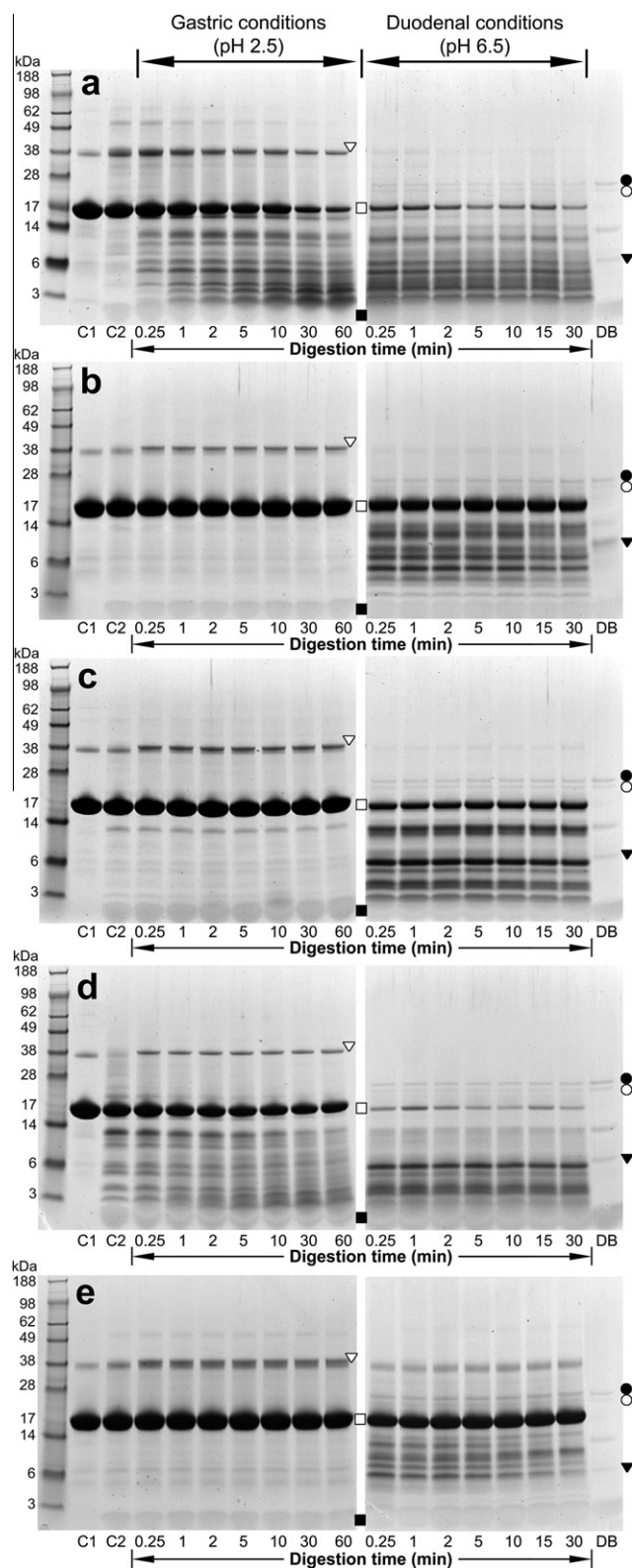


Fig. 4. SDS-PAGE (reducing conditions) characterisation of *in vitro* gastric followed by duodenal digestion of purified β -Lg heat-treated samples. Before digestion the protein was heated at 70 °C for 24 h at pH 6.5 (a), pH 5.2 (b), pH 4.8 (c), pH 2.5 (d) or 85 °C for 0.5 h at pH 5.2 (e). The abbreviations are: C1, control sample before heat-treatment; C2, control sample after heat-treatment; DB, duodenal background (i.e. a mix of trypsin, chymotrypsin, phosphatidylcholine (PC) and Bowman-Birk trypsin/chymotrypsin inhibitor; all at concentrations used in the active duodenal samples). The symbols indicate the bands corresponding to: (∇) pepsin, ($\square$) β -Lg, ($\blacksquare$) PC, ($\bullet$) chymotrypsin, ($\circ$) trypsin, ($\blacktriangledown$) Bowman-Birk trypsin/chymotrypsin inhibitor.

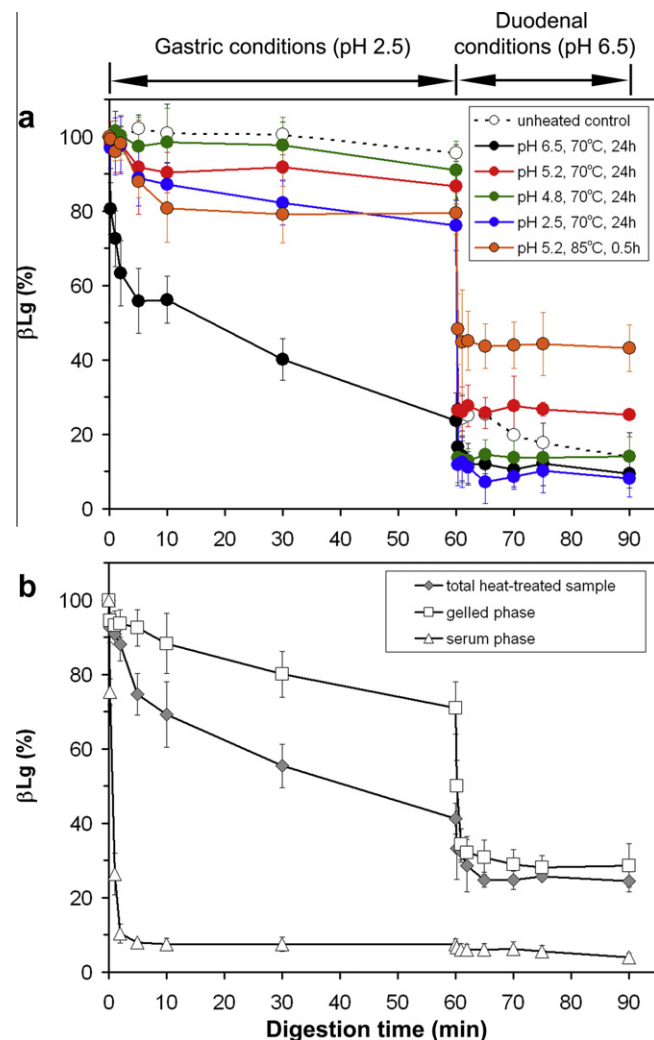


Fig. 5. Quantification of the SDS-PAGE results: (a) digestion of β -Lg in heat-treated samples from the purified β -Lg (see Fig. 4; the heating conditions given in the legend); (b) digestion of β -Lg in heat-treated WPI samples (see Fig. S5). Heated WPI samples were used either directly after the heat-treatment ('total heat-treated sample') or after additional separation of the samples into a 'gelled phase' and a soluble ('serum') phase, following the heat treatment. Initial 100% of β -Lg refers to the amount of the monomeric β -Lg remaining after the heat-treatment.

It is not possible to follow the kinetics of enzyme action using the data in Fig. 5 but the data does highlight the fact the β -Lg shows a range of behaviour in the same sample. This is especially apparent in the simulated duodenal digestion where in each case a fraction of the sample was very quickly degraded, probably in seconds, whilst the remaining fraction (with the exception of the unheated control) appeared completely resistant. With the exception of the sample heated at pH 6.5, the same behaviour appears through simulated gastric digestion.

In addition to assessing the impact of heating on purified β -Lg, we treated WPI and followed the digestion of β -Lg from that. The applied heat-treatment produced a firm white gel (Fig. S2f) made of stranded nanometre sized (100–200 nm) microgel particles (Fig. 6a). The digestion results are shown in Fig. 5b and reveal that the total heat-treated WPI sample (i.e. the sample obtained directly after the heat-treatment) was significantly degraded through simulated gastric digestion but was fairly resistant to simulated duodenal conditions, with over 20% β -Lg content remaining undigested. The overall structure of the gel was destroyed by digestion but the protein that remained was present in the form of microgel particles closely resembling, in shape and size, the microgels that

the initial heat-treated sample was composed of (Fig. 6). Samples of heated WPI were also split into their component gel and serum phases and then digested separately. The results show that the gelled protein was very much more resistant to digestion than the serum phase, which was quickly degraded during the simulated gastric phase to leave only a small proportion of the β -Lg intact. The SDS-PAGE analysis of digestion of the WPI samples is shown in Fig. S5 and shows the similarity in behaviour of both the β -Lg and the α -lactalbumin in WPI.

4. Discussion

It is well known that the structure and properties of protein gels depends on the conditions under which they are formed (van Vliet et al., 2004). Indeed a scan of the literature can show that the particle sizes we have produced are typical for the conditions used. For example, pH 6.5 and a concentration of 40 g/l is similar to conditions used by Roefs and de Kruif who showed that the particles formed were between 30 and 40 nm in size (Roefs & de Kruif, 1994), which is in reasonable agreement with the findings presented here. However, this is the first time that these structural differences have been assessed in terms of susceptibility of the protein to proteolysis under conditions simulating those found in the upper GI tract.

Previous work has shown that unheated β -Lg was not affected by pepsin up to 60 min with no change in the protein bands over time (Mandalari et al., 2009). However, this is not true of heated β -Lg, although the heating conditions may have a strong influence on pepsin resistance. β -Lg has its main thermal transition at 75 °C and has an isoelectric point of pH 5.2–5.6, depending on the variant (Lyster, 1972), although in our samples heated to 70 °C for 24 h the value was closer to pH 4.8. When β -Lg is heated to 70 °C, which is below the main transition temperature, a proportion of the protein partially unfolds and thus has an increased propensity to aggregate. In the experiments described above, the sizes of the aggregates formed depended on the net charge on the β -Lg; the smaller the charge the larger the aggregates or gel particles formed. When heated at pH 2.5 only 70% of the β -Lg was incorporated into the gel and this, in combination with the high net charge led to the formation of a weak fine stranded gel. At pH 6.5, although the net charge was slightly lower, 90% of the protein

was incorporated into the gel making it much stronger than that formed at pH 2.5, even though the gel particle size was similar. These fine stranded gels presented a large surface area and consequently were highly susceptible to proteolysis. Indeed, the pH 6.5 sample was particularly susceptible to pepsin hydrolysis with only 24% remaining at the end of the simulated gastric phase, although it is unclear why this should be.

Closer to the isoelectric point of β -Lg the net charge was very low and this led to the formation of larger aggregates at pH 4.8 and 5.2. These formed very quickly, but only incorporated about half the available protein and consequently failed to form a continuous network. This was not important for the general outcome as enzymes are equally able to diffuse through continuous gels. The aggregates showed little or no intermolecular β -sheet and whilst both gels were quite resistant to pepsinolysis, the gel at pH 4.8 showed only limited resistance to simulated duodenal digestion. When β -Lg was heated to 85 °C at pH 5.2, large aggregates containing significant amounts of intermolecular β -sheet were quickly formed and at this temperature more than 90% of the available β -Lg was incorporated into the gel, forming a highly elastic network. Both gels formed at pH 5.2 showed resistance to proteolysis but the gel formed at 85 °C was particularly resistant with more than 40% of the β -Lg remaining intact at the end of digestion. There is some evidence that the formation of dense beta structures, such as amyloid can infer proteolytic resistance (Soto & Castano, 1996) but this may not hold for all intermolecular beta structures. The β -Lg in WPI has been thermally treated as part of the preparation process. Thus, one might expect to see it unfold and aggregate very quickly once heated to 85 °C and the rheological data shows this to be the case. In contrast to the β -Lg, a moderately fine stranded gel was formed with gel particles of about 100–200 nm. The smaller gel particle size may also have contributed to the reduced resistance to digestion. Less than 30% of the β -Lg from the WPI sample remained intact after digestion as opposed to more than 40% for the β -Lg treated alone.

In this work we set out to identify the nano- and micro-scale properties of protein gels that control their resistance to proteolysis under simulated upper GI conditions. It is clear from Fig. 5 that gelation of β -Lg can impart protease resistance, although pepsin resistance did not correlate well with trypsin and chymotrypsin resistance. With the exception of the sample heated at pH 2.5, which underwent acid hydrolysis, persistence through simulated

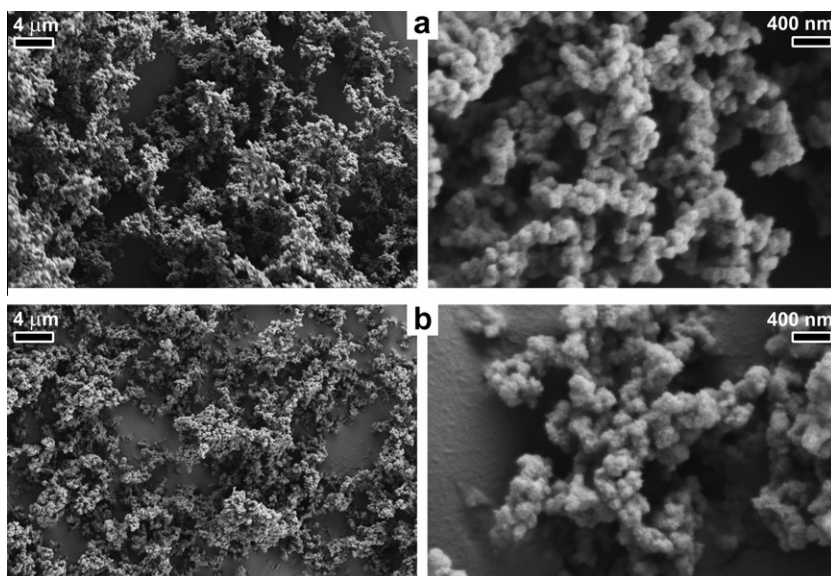


Fig. 6. SEM images of WPI gel ('total heat-treated sample') before (a) and after (b) gastro-duodenal digestion.

gastric digestion was strongly correlated with the size of the gel particles. This suggests that pepsin was only able to digest β -Lg on the surface of the aggregates and that in most cases the peptides formed remained part of the aggregates or were only slowly removed. This appears to be partially confirmed by the images of WPI before and after digestion as shown in Fig. 6. Assessing the main factors that determined subsequent β -Lg persistence through simulated duodenal conditions is more complex and there is no clear correlation with any of the parameters measured, although the stronger gels formed at 85 °C were quite resistant. These ideas are supported by previous work on iron release from whey protein gels (Remondetto et al., 2004). In their simulated gastric step, a small amount of iron (20%) was released from both gel types, indicating that in both fine stranded and particulate networks a significant fraction of the gel remains in the low-pH environment of the stomach. However, fine stranded gels released larger amounts of iron in the intestine, where most intracellular iron uptake occurs, thus favouring increased iron absorption. This again confirms the greater persistence of particulate gels over fine stranded gels in conditions simulating the small intestine.

In this work we have used a range of thermal and pH conditions to form weak gels with structures ranging from fine stranded to particulate. We have then exposed these gels to proteolysis conditions simulating those in the upper GI tract. The results show that fine stranded gels are digested to a greater extent than the particulate gels although this was complicated by acid hydrolysis of the samples heated at pH 2.5. In addition, heating the β -Lg or WPI to 85 °C for 30 min at pH 5.2 formed particulate gels with greater resistance to proteolysis than heating to 70 °C for 24 h. This clearly shows the potential of protein gels as delivery systems or for use in modulating uptake.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2012.04.018>.

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