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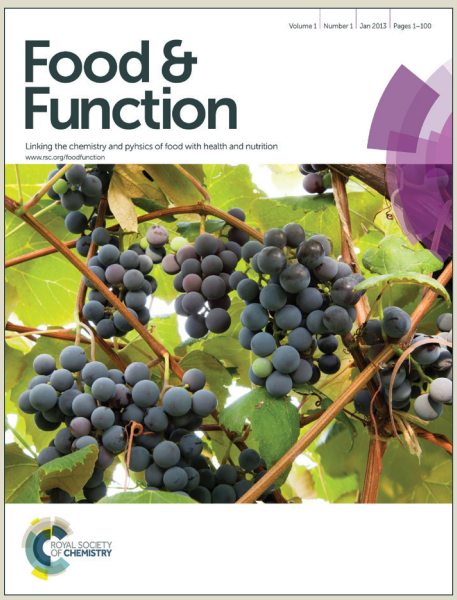

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Influence of heat and shear induced protein aggregation on the *in vitro* digestion rate of whey proteins

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Abstract

Protein intake is essential for growth and repair of body cells, the normal functioning of muscles, and health related immune functions. Most food proteins are consumed after undergoing various degrees of processing. Changes in protein structure and assembly as a result of processing impact the digestibility of proteins. Research in understanding to what extent protein structure impacts the rate of proteolysis under the human physiological conditions has gained a considerable interest. In this work, four whey protein gels were prepared using heat processing at two different pH values, 6.8 and 4.6, with and without applied shear. The gels showed different protein network microstructures due to heat induced unfolding (at pH 6.8) or lack of unfolding, thus resulting in fine stranded protein network. When shear was applied during heating, particulate protein networks were formed. The differences in the gel microstructures resulted in considerable differences in their rheological properties. An *in vitro* gastric and intestinal model was used to investigate the resulting effects of these different gel structures on whey protein digestion. In addition, the rate of the digestion was monitored by taking samples at various time points throughout the *in vitro* digestion process. The peptides in the digesta were profiled using SDS-polyacrylamide gel electrophoresis, reversed-phase-HPLC and LC-MS. Under the simulated gastric conditions, whey proteins in structured gels were hydrolysed faster than native proteins in solution. The rate of peptides released during *in vitro* digestion differed depending on the structure of the gels and extent of protein aggregation. The outcomes of this work highlighted that changes in the network structure of the protein can influence the rate and pattern of its proteolysis under gastrointestinal conditions. Such knowledge could assist the food industry in designing novel food formulations to control the digestion kinetics and the release of biologically active peptides for desired health outcome.

Introduction

Whey proteins, which consist principally of β-lactoglobulin (β-Lg), α-lactalbumin (α-La) and bovine serum albumin, have globular structures. Whey proteins are widely used as food ingredients because of their versatile functional properties such as viscosity building, foaming, emulsifying and gelling.[1] Enzymatic hydrolysis of whey proteins can further modify and/or improve functional properties which include increased solubility, decreased viscosity, and significant changes in foaming, gelling, and emulsifying properties compared to those of native proteins.[2] Such modification can offer further interesting food applications for whey proteins.

Research has highlighted potential health enhancing benefits of intact whey proteins and hydrolysates produced from whey proteins.[3] The biological activities or health enhancing benefits attributed to peptides originating from whey proteins are antihypertensive or ACE inhibitory,[4] opioid,[5] bactericidal,[6] immunomodulating[7] and hypocholesterolemic.[8] In addition, peptides originated from whey proteins can also confer increased satiety, protect against muscle-protein loss, enhance muscle-protein synthesis, and improve glycemic control.[9]

Most food proteins undergo structural transformation/denaturation during processing or home cooking. The native structures of proteins are altered as a result of physical treatment such as heating and shearing, chemical factors such as pH and ionic strength or presence of other ingredients. Such changes may have both desirable and undesirable effects on protein digestibility.[10] For example, mild heating can improve digestibility by partially denaturing the proteins, but severe heat treatment may reduce protein digestibility by cross-linking, racemization, and the Maillard reaction.[11] Further, alteration of native protein structures can result in changes in protein digestibility and subsequently the availability of essential amino acids.[12] A relative resistance of whey proteins to proteolysis is generally explained by a compact tertiary structure of the protein that restricts susceptible peptide bonds from enzyme cleavage. A recent study by Barbé *et al.*

(2013)[13] on the digestive contents and plasma samples taken over a 7 h-period after meal consumption showed that gelation of milk by coagulation slowed down the outflow of the meal from the stomach and the subsequent intestinal absorption of amino acids which decreased their bioavailability in peripheral blood. Heating of milk led to enhanced susceptible of of β-lactoglobulin (β-Lg) and caseins to protein hydrolysis in the stomach, resulting in a higher plasma leucine concentration compared with ingestion of unheated milk.[13] Heat treatments applied to milk for the manufacture of pasteurized, sterilized milks and yogurts also resulted in an increased digestibility of β-Lg.[14] Heat-induced β-Lg digestion by pepsin has also been shown by Peram *et al.* (2013) in an *in vitro* study.[15]

The process of food digestion begins with the breakdown of ingested food in the human gastrointestinal tract via a multitude of mechanical and enzymatic processes to ensure that the components and nutrients become available for absorption. The increased evidence from studies using *in vitro* models or clinical data has provided a basis for this new area of research regarding the role of food structure on the nutritional properties of conventional and functional foods. It is now clear that the composition and processing of foods need to be carefully balanced to ensure optimal nutritional values of food.[16] The objective of this study was to investigate the influence of various gel microstructures (formed by changes in pH, with or without shear application during heat induced gelation) on the *in vitro* digestion of whey proteins, as in the rate and type of released peptides.

Materials and methods

Materials

Whey protein isolate (Fonterra WPI90; PROTEIN 87.3%, moisture content 5.5%) was purchased from Rogers & Company Foods Pty Ltd (Hampton, Victoria, Australia). Bile salts

extracts, porcine pepsin (P7000, 882 U/mg), pancreatin (P7545, Porcine, USP × 8) and bile extract porcine (B8631, total bile salt content = 49 wt%; with 10-15% glycodeoxycholic acid, 3-9% taurodeoxycholic acid, 0.5-7% deoxycholic acid; phospholipids 5 wt%) were obtained from Sigma-Aldrich, Australia. Acetonitrile and trifluoroacetic acid (TFA) used in the preparation of mobile phase for the reverse phase high performance liquid chromatography (RP-HPLC) were, both HPLC grade (Merck Millipore, Kilsyth, VIC, Australia). All other solvents and reagents were of analytical grade.

Whey protein gel preparation

WPI 10% (w/v; total solids) solution was prepared by dissolving 10.6 g of WPI powder in ~ 90 mL deionised water and stirred constantly at 4 °C overnight to disperse the powder completely. The final volume of the solution was made to 100 mL with deionised water. The pH of the solution was adjusted to 6.8 or 4.6 by slow addition of 0.1M NaOH or 0.1M HCl and degassed. The non-sheared gels were prepared by heating the samples in a water bath at 90 °C for 10 min. The sheared gels were prepared using a Rapid Visco Analyser (RVA4, Newport Scientific, Australia). The protein solutions were heated from 25 to 90 °C with a gradient of 5 °C/min and held at 90 °C for 10 min. The protein mixture was sheared at 500 rpm (equivalent to ~52/s) during the heating process. The gels were then cooled down to 25 °C.

Confocal laser scanning microscopy

Imaging of gel structures was carried out at room temperature with a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) using a HCX PL APO 63× objective. The protein was fluorescently labelled prior to gelation (in the RVA or waterbath) by addition of Fast Green (0.4% in water) to the samples for a final dye concentration of 0.01%. The fluorescent dye was excited at 633 nm and the reflected emitted light was collected at 646-726 nm.

Rheology measurements

The rheological properties of whey protein gels were measured using an Anton Paar-Physica stress control rheometer (MCR 301, Anton Paar Physica, Physica Meßtechnik GmbH, Stuttgart, Germany) with a vane geometry (cup inner diameter 28.9 mm, 6 vane blades; width 10 mm, length 16 mm). To avoid disruption of the gel structure, the gels were created directly in the rheometer using conditions similar to those used to prepare the gels for in vitro digestion. For non-sheared gels, the whey protein solution (approximately 10 mL) was loaded to the cup after adjustment of pH to 4.6 or 6.8. The gel was formed by heating at 5 °C/min to 90 °C. The sample was then held for 10 min at 90 °C, cooled to 20 °C at a cooling rate of 5 °C/min and held for another 2 hr to allow the gel to set. For the sheared gels, the same heating/cooling profile was used but under application of rotational shear at a constant shear rate of 52 /s.

Oscillation rheology measurements were carried out at 20 °C, first a frequency sweep from 0.01 to 10 Hz at a constant strain of 0.1% followed by a strain sweep from 0.01 to 100% at a constant frequency of 1 Hz. A resting time of 10 min was allowed between the two sweep measurements. Duplicate measurements were taken for separately prepared whey protein solutions.

In vitro digestion

Protein digestion was carried out using a two-step *in vitro* protocol. Samples of WPI solution (10% protein; 1 g) or gels (10% protein; 1 g) were mixed with 2.5 mL of simulated gastric fluid, which consisted of 2 mg/mL NaCl and 4.5 mg/mL pepsin at pH 1.9,[17-19] and incubated at 37 °C over 2 h under constant agitation. An aliquot (90 µL) of the digesta (for analysis of breakdown products) was withdrawn at various time intervals within 2 h and mixed immediately with 10 µL 10% (v/v) TFA solution to terminate the enzymatic reaction. A parallel gastric digestion was carried out without removal of the digesta. After 2 h, the pH of this digesta was adjusted to 6.8 using 0.1 M NaOH. Simulated intestinal fluid (SIF, 11.25 mL) was added to achieve a final composition of 10

mM $CaCl_2$, 12 mM mixed bile salts, 150 mM NaCl and 4 mM tris(hydroxymethyl)aminomethane buffer, followed by addition of 3.75 mL of Tris buffer and 200 µL of SIF containing pancreatin.[17-19] The digestion was maintained at 37 °C over 2 h under constant agitation. An aliquot (90 µL) of the digesta was withdrawn at various time intervals and mixed with 10 µL 10% (v/v) TFA solution to terminate the enzymatic reaction.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All chemicals and gels used for SDS-PAGE analysis were purchased from Life Technologies Australia Ltd (Mulgrave, VIC, Australia). Samples taken at different times of the gastric and intestinal digestion were mixed with SDS-PAGE buffer (NuPAGE SDS PAGE sample buffer) and reducing agent (NuPAGE sample reducing agent). The sample mixtures were heated in a water bath at 90 °C for 10 min and centrifuged at 14,600 g for 10 min. The supernant (5 µL of the gastric or intestinal digesta containing 12 µg protein) was loaded onto a PAGE gel (Novex Tris Glycine precast gradient gel, 4-20% polyacrylamide). The gel electrophoresis was run using tris-glycine SDS running buffer (Novex Tris-Glycine SDS running buffer) at 200 V for 35 min. The gel was then stained using a Coomassie stain (SimplyBlue Safe Stain). A prestained molecular weight marker comprised the following mix of proteins: myosin (188,000 Da), phosphorylase B (98,000 Da), BSA (62,000 Da), glutamic dehydrogenase (49,000 Da), alcohol dehydrogenase (38,000 Da), carbonic anhydrase (28,000 Da), myoglobin-red (17,000 Da), lysozyme (14,000 Da), aprotinin (6,000 Da) and insulin B chain (3,000 Da). Stained gels were imaged using Gel Imaging System (Syngene, Frederick, MD, USA).

Reverse phase high performance liquid chromatography (RP-HPLC)

All WPI solution and gel digesta samples were centrifuged (25000 g, 20 min, 4 °C) to remove large peptides and intact milk proteins. The supernatant containing soluble proteins and peptides were filtered through a 0.45 µm filter before the sample was injected to the column. HPLC

was performed using an automated Thermo Finnigan Surveyor Plus system fitted with a widepore C18 reversed phase Aeris column (particle size 3.6 µm, pore size 300 Å, 150 mm x 2.1 mm) and a guard column (10 mm x 2.1 mm). The column temperature was maintained at 35 °C. Peptide separation was achieved with a linear gradient of 2% to 70% solvent B (0.08% TFA in acetonitrile) in solvent A (0.1% TFA in water) over 65 min. The flow rate was maintained at 150 µL/min. The eluate was monitored at 214 nm using a photodiode array detector.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

The samples were analysed by LC-MS/MS on a Shimadzu Nano HPLC directly coupled to an AB/Sciex 5600 TripleTOF mass spectrometer. 10 µL of each sample was injected after dilution in 0.1% formic acid to give the same starting concentration. An Agilent Zorbax C18 column (3.5 µm, 150 mm x 75 µm) was used for peptide separation using a linear gradient from 2-40% solvent B in solvent A over 30 min (flow rate of 300 nL/min) where solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in 90% acetonitrile. The eluate was directed into the nanoelectrospray ionisation source of the TripleTOF™ 5600 system (AB/Sciex, Foster City, USA). Data were acquired in information dependent acquisition (IDA) mode. The IDA method consisted of a high resolution TOF-MS survey scan followed by 20 MS/MS in a second with a maximum accumulation time of 50 ms. First stage MS analysis was performed in positive ion mode over the mass range *m/z* 300-1800 with a 0.5 s accumulation time. The ionspray voltage was set to 2400 V, the curtain gas was set to 25, the nebuliser gas to 12 and the heated interface was set to 180 °C. Tandem mass spectra were acquired on precursor ions that exceeded 120 cps with charge state 2 to 5. Spectra were acquired over the mass range *m/z* 80-1800 using rolling collision energy (CE) for optimum peptide fragmentation. Precursor ion masses were excluded for 8 s after two occurrences.

ProteinPilot™ 4.1.46 software (Applied Biosystems) with the Paragon Algorithm was used for the identification of proteins. Tandem mass spectrometry data was searched against *in silico* (no enzyme selected) digests of bovine proteins of the Uniprot database (version 2012/12; 67,004

sequences) or a custom database comprised of β-Lg and α-La. All search parameters were defined as no cysteine alkylation, with no digestion enzyme selected (allows cleavage at any point on protein sequence). Modifications were set to the "generic workup" and "biological" modification sets provided with this software package, which consisted of 126 possible modifications, for example, acetylation, methylation and phosphorylation. The generic workup modifications set contained 51 potential modifications that might occur as a result of sample handling, for example, oxidation, dehydration and/or deamidation.

Results and discussion

Differences in the microstructures of whey protein gels

The molecular assembly of whey proteins into aggregates and networks is dependent on the conditions during gel formation. Factors such as pH, protein concentration, co-solute, heating profiles and application of shear, all play a role in determining the final microstructure of whey protein gels.[20-25] Controlling the pH of the whey protein sample during the heating step generates opportunity to design network structures with varying morphology. If a pH close to the p*I* of the protein is chosen, the heat denatured protein will assemble into a particulate gel and at a pH close to neutral, the resulting heat set network structure will be more homogenous and fine stranded.[20] In order to investigate the influence of protein supramolecular structural differences on the accessibility of proteases to cleavage sites within the protein sequence and the resulting composition and size of the peptides being generated, a range of structures with the same protein concentration were created by heating the whey protein at different pH (4.6 and 6.8), with or without applied shear. The network structures of these gels were visualised at the micrometer length scale using confocal laser scanning microscopy and shown in **Fig. 1**. For the gel produced at pH 6.8 without shear, a fine stranded network microstructure formed, consisting of linked protein

assemblies (**Fig. 1a** and **1b**). The microstructure of the sheared gel produced at pH 6.8 (**Fig. 1c** and **1d**) consisted of irregular large protein agglomerates (100-500 μm wide) dispersed in the serum phase (**Fig. 1c**). Within the agglomerates fine stranded fibrous structures similar to the non-sheared sample could be observed (**Fig. 1d**). On the other hand, the microstructure of the whey protein gel prepared at pH 4.6 without shear was dominated by spherical protein aggregates (**Fig. 1e and 1f**). These protein aggregates were closely packed forming a network with pores 10-50 μm wide. When shear was applied during gelation, the particulate network was broken into larger agglomerates in the size range of 50 μm, which contained many of the small protein aggregates (**Fig. 1g** and **1h**). The gels prepared at pH 4.6, with or without shear, showed a similar internal structure assembled from 2 μm spherical protein particles. However, the small spherical particles in the sheared gel (i.e. particulate agglomerates) appeared to be more closely packed than those in the non-sheared gel.

The heat induced gelation of WPI involves the formation of molecular linkages through disulphide and hydrogen bonds, Van der Waals, hydrophobic and electrostatic interactions. The extent of the various interactions depends on the conditions, where both pH and salt concentration have a major impact on the surface charge and electrostatic repulsion of the protein molecules. When the surface charge and electrostatic repulsion are reduced, the protein molecules form primary aggregates (50-300 nm) that can associate further into larger polydisperse agglomerates which, if the protein concentration is high enough, can then be connected to form a network,[25] as shown in **Fig. 1g** and **1h**. In the conditions where strong electrostatic repulsions are present, that is at pHs away from the isoelectric point of the whey protein, the gels formed are transparent and have a fine-stranded structure,[26] as shown in **Fig. 1a** and **1b**. The differences in the WPI gel microstructures observed are manifestation of changes of whey proteins at the molecular level. Physico-chemical parameters applied during the protein gelation process have major consequences for the forces acting at the short length scale or molecular level which leads to changes in spatial arrangement of protein strands and junction points due to interplay of inter- and intra-molecular

interaction. Such changes to the interactions at the molecular level and the resulting differences in the structure of the protein assemblies could impact on the accessibility of specific peptide bonds to enzymatic digestion.

Rheological properties of whey protein gels

The viscoelastic properties of heat induced whey protein gels formed at pH 4.6 and 6.8 with and without shear were measured at 20 °C. The storage modulus G', loss modulus G" and tan δ values of the protein networks as a function of frequency in oscillation are shown in **Fig. 2a** and **2b**, for the protein systems prepared at pH 4.6 and 6.8, respectively. All protein samples displayed a weak gel behaviour, with G' higher than G" at all the frequencies, with G" lower than G' by less than 10-folds.[27] However, the storage modulus G' and loss modulus G" were lower for the gels formed at pH 6.8 than the gels formed at pH 4.6, indicating differences in the protein interactions at these two pHs. For the gels formed at the two pHs, application of shear reduced the gel strength as indicated by the lower G' and G" values compared with the gels prepared without shear. Interestingly the phase angle, tan δ, for both the non-sheared and sheared samples is ~0.2 and independent of frequency below 1 Hz. This implies that both systems behaved as protein networks and it is likely that the mesoscale supramolecular structure is the same at the respective pH, regardless whether the protein gels were formed with or without shear. This suggests that the protein agglomerates formed, during the combined heat and shear process, are interacting and are of sufficiently high phase volume to form a space filling network. This interpretation is supported by the CLSM images of the sheared gels showing large agglomerates of protein aggregates for the sample prepared at pH 6.8 (**Fig. 1c**) compared to a homogenous network when prepared without shear (**Fig. 1a**). The formation of larger protein agglomerates in the sheared gel (**Fig. 1g**) compared with a particular network in the non-sheared gel at pH 4.6 (**Fig. 1e**) would also reduce the protein interactions.

The gel was largely formed by protein aggregates at pH 4.6 (**Fig. 1e**), although there appears to be more close range interactions between and within the protein aggregates as shown by the higher G' and G" values compared with the fine stranded network formed at pH 6.8, the (non covalent) interactions between the protein aggregates was weaker than the covalent links in the stranded network. Not surprisingly, the sheared gels started to deform and flow at even earlier strain (5% and 1% respectively for pH 6.8 and 4.6 gel) due to disruption of protein interactions caused by the shear applied during gel formation and the resulting network architecture of interacting protein agglomerates.

The rate of protein digestion as a function of protein assembly

Whey proteins in solution and structured gels were subjected to enzymatic digestion using a simulated gastric and intestinal model[17-19] and the protein breakdown was followed by SDS-PAGE and RP-HPLC.

The SDS-PAGE results showed that the digestion of β-Lg and α-La, the most abundant proteins in whey protein isolate, in solution state was relatively slow, as indicated by little change in the intensity of the β-Lg and α-La bands during the 2 h gastric digestion (**Fig. 3a**, lanes 3-7). However several low molecular weight bands, below the protein bands, were visible throughout the 2 h gastric digestion, indicating that a small amount of protein had been digested. This was confirmed by the RP-HPLC results showing an intact β-Lg protein peak at the end of 2 h gastric digestion, together with a range of peptides (**Fig. 4a**).

In contrast, for the structured WPI gels, both β-Lg and α-La were relatively rapidly digested by pepsin as the protein bands faded away from very early stages of gastric digestion (**Fig 3b-e**, lane 3) and with little or no proteins visible at the end of the gastric digestion (**Fig. 3b-e**, lane 7). This was accompanied by the appearance of a large number of lower molecular weight bands, migrating faster than the parent whey proteins. Proteins in the sheared gels (**Fig. 3c** and **3e**)

appeared to be digested slightly slower than the non-shear gels (**Fig. 3b** and **3d**). This could be due to the differences in the gel microstructure where the denser agglomerates for the sheared gels, as seen in **Fig. 1**, might have slowed down the enzyme diffusion into the gel particles due to steric hindrance. Recent work of Macierzanka *et al.* (2012) also showed that firm particulate WPI and β-Lg gels persisted longer during simulated gastro-duodenal proteolysis.[28]

Proteolysis of whey proteins and peptides in intestinal conditions progressed rapidly for all samples as shown by the complete disappearance of the protein bands on SDS-PAGE gels after 4 min of simulated intestinal digestion (**Fig 3** a-e, lanes 8-12). The fine stranded network of the pH 6.8 gel showed the fastest intestinal digestion with no protein breakdown products left in the SDS-PAGE gel after 1 min of digestion. Similar to the observation in the gastric digestion, the intestinal digestion of the sheared structured WPI gels progressed slower than the non-sheared gels at both pHs. The intestinal digestion of WPI in solution (**Fig. 3a**, lanes 8-12) was the slowest with more peptide bands present compared to the structured gels. After 2 h of simulated intestinal digestion, no peptide bands could be seen for any of the protein samples indicating that the peptides had all been hydrolysed to sizes smaller than ~3,000 Da.

Fig. 4 shows the RP-HPLC peptide profiles of digested samples taken at 10 min gastric phase and 4 min into the intestinal phase. The peptide profiles of digesta samples were complex showing large number of peaks which indicates extensive proteolysis of the whey protein in gastric and intestinal conditions. Overlaid RP-HPLC chromatograms of gastric phase digesta samples clearly showed that β-Lg and α-La appearing at ~55 min as large peaks in the solution sample in gastric condition (**Fig. 4a,** top line). The proteins were still detectable 4 min into the intestinal phase of digestion (**Fig. 4b**, top line). However, for all gel samples the whey proteins were completely digested within 10 min in the gastric phase, which confirms the observations made from the SDS-PAGE analysis (**Fig 3 a-c**). Some differences were observed between the peptide profiles of whey protein solution and gels at both gastric (**Fig. 4a)** and intestinal phases (**Fig. 4b).** Differences in

296 peptide patterns between gels made at differing pH 4.6 or 6.8 can also be seen in **Fig 4a**. Relatively
297 small differences in peptide patterns were observed in whey protein gels with respect to whether
298 gels were sheared or non-sheared.

299 Resistance of native β-Lg to pepsin hydrolysis is primarily attributed to its unique structural
300 stability at low pH.[29-32] The low digestibility of whey proteins from raw milk is linked to their
301 structure, as they are stabilised by a number of disulphide bonds.[31, 33] Most of the hydrophobic
302 amino acid residues, which are potential cleavage sites for pepsin, are buried inside the hydrophobic
303 core and thus not readily accessible. A variety of stresses applied (heating, high pressure treatment,
304 addition of alcohols, and esterification) have been reported to increase the susceptibility of β-Lg to
305 hydrolysis by pepsin.[34-37] These treatments induce conformational changes in β-Lg, resulting in
306 increased exposure of peptic cleavage sites and thus increased susceptibility of the protein to pepsin
307 action. The recent work by Peram *et al.* (2013) clearly showed that stable high molecular weight
308 aggregates (*e.g.* pentamers, tetramers, and trimers) generated during heat-induced aggregation of β-
309 Lg were digested relatively rapidly compared with native and non-native dimers.[15] It is interesting
310 to note that the dimers formed during the early stages of heating (*e.g.* 5 min of heating) were quite
311 resistant to digestion, whereas those formed after 120 min of heating were easily digested by
312 pepsin. The dimers formed during the early stages of unfolding probably retained some of their
313 secondary and tertiary conformations and the pepsin cleavage sites remained largely buried in the
314 hydrophobic core and thus were not accessible to pepsin. Heating for a longer time (*e.g.* 120 min)
315 resulted in significant changes in the secondary and tertiary structures of the dimers and
316 consequently promoted their digestibility with pepsin. Macierzanka *et al.* (2012) studied *in vitro*
317 gastrointestinal digestion of 4% β-Lg and WPI gels, made using a range of thermal (70 and 85 °C
318 for varying times) and pH (pH 6.5, 5.2, 4.8 or 2.5) conditions, which resulted in formation of weak
319 gels with structures ranging from fine stranded to particulate.[28] Their results showed that fine
320 stranded gels (which are formed at pH 6.5 and 2.5) are digested to a greater extent than the

particulate gels (which are formed at 5.2 and 4.8). 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. The results of a recently published study by Zhang and Vardhanabhuti (2014)[38] also concurred with the observations made by Macierzanka *et al.* (2012).[28] However, in this current study, it appears that all β-Lg was digested much faster in gels of both pHs than in solution when the gels were prepared at 90 °C for 10 min, although there were some differences in the extent of β-Lg digestion between the sheared and non-sheared gels (**Fig. 3**).

Peptides released during digestion

The peptides produced at different stages of simulated gastric and intestinal digestion were further analysed by liquid chromatography tandem mass spectrometry. The peptide amino acid sequences determined experimentally were matched with known/published primary sequences of β-Lg and α-La to deduce the origins and are summarised in Tables 1 and 2, respectively. Peptides identified in the WPI solution and structured gels, were also presented as line diagrams on respective parent whey proteins, namely β-Lg and α-La, marked with the known cleavage specificities of digestive proteases (pepsin, chymotrypsin and trypsin; ExPASy Peptide Cutter, Swiss Institute of Bioinformatics : http://web.expasy.org/peptide_cutter/), see **Fig. 5** and **6**.

β-lactoglobulin

A total of 67 peptides originating from β-Lg were identified in the digesta samples of WPI solution and structured gels. Most of them were from the terminal regions L_1-K_{60} and L_{122}-I_{162} of β-Lg (**Fig. 5**). Only a few peptides originated from the middle region W_{61}-C_{121}. It appears that the region 61-121 is fairly resistant to hydrolysis by proteolytic enzymes in *in vitro* gastric and intestinal model systems. This region contains 4 out of 5 Cys residues of β-Lg which are involved in intra- and inter-molecular disulphide linkages to maintain the tertiary structure of the native protein, render potential cleavage sites of pepsin, chymotrypsin and trypsin inaccessible. More than

half of the peptides produced in the gastric digestion were common for β-Lg in solution and in structured gels and they were further hydrolysed in the intestinal phase of digestion leading to the disappearance of same peptides. However, there were a few key differences in the peptide patterns produced during the digestion between WPI in solution and structured gels. Eighteen peptides were found in the WPI solution digesta which was not in any of the structured gels, whereas another 14 peptides were found present in the digested gels but absent in WPI solution.

Specific peptides identified only in the WPI solution gastric digesta samples were β-Lg f2-20, f9-19, f9-20, f32-42, f71-82, f123-131, f123-133 and f131-149 (**Table 1**). The production of these peptides clearly showed that specific peptide bonds in β-Lg sequence Y_{20}-S_{21}, K_{70}-I_{71} and D_{130}-E_{131} were only hydrolysed from the proteins in WPI solution because the peptides starting or ending with these specific sequences were not found in the digesta of structured gels (**Table 1** and **Fig. 5**). On the other hands, four peptides were identified in the gastric phase digestion of structured gels, but not in WPI solution. These were β-Lg f1-19, f41-51, f41-55 and f133-146, **Fig. 5**). There were also some differences in terms of the presence/absence of these peptides at specific time points and between the various structured gels (**Table 1**). Peptides β-Lg f1-19 and f41-51 were only produced from particulate networked gel (pH 4.6 non-sheared). Peptide β-Lg f133-146 was produced at the end of gastric phase digestion of fine stranded network and stranded gel aggregate gels (pH 6.8, non-sheard and sheared) and rapidly degraded further in intestinal phase of digestion. In contrast, peptide β-Lg f41-55 was produced at the beginning of gastric phase digestion of both fine stranded and particulate networked gels (pH 4.6 and 6.8, non-sheared) and further hydrolysed rapidly as the gastric digestion progressed.

In the intestinal digestion phase, seven peptides were identified in the WPI solution digesta which were not found in the digesta of structured gels. They were β-Lg f27-40, f27-41, f27-42, f30-41, f40-57, f118-138 and f125-138 (**Fig. 5**). Production of these peptides from β-Lg required

hydrolysis of specific peptide bonds, *i.e.* A_{26}-S_{27}, I_{29}-S_{30}, L_{39}-R_{40}, S_{117}-L_{118}, R_{124}-T_{125} and K_{138}-A_{139}. Among these peptide bonds only L_{39}-R_{40} and R_{124}-T_{125} / K_{138}-A_{139} are known cleavage sites of chymotrypsin and trypsin, respectively. These peptides were not found in the intestinal digestion of structured gels. This could be due to two reasons: (a) peptides produced were rapidly further hydrolysed to peptides < 5 amino acids which could not be identified by mass spectrometry analysis employed in this work, or (b) the structure of proteins in the stranded and agglomerate network hindered the accessibility of enzymic to specific bonds. On the other hand, 10 peptides were produced in the intestinal digestion of structured gels, but were not found in the digested WPI solution. Six of these peptides, namely β-Lg f42-51, f43-51, f43-56, f125-135, f125-136 and f125-137, were produced very late during the intestinal phase of the gel digesta and were detected in all four types of gels. Further, three peptides (β-Lg f1-20, f41-60 and f43-59) were identified only in particulate networked gels which were produced at pH 4.6 (non-sheared and sheared). It is possible that the order of hydrolysis of the K_{60}-W_{61} bond by trypsin is different at the acidic pH. One peptide (β-Lg f82-92) found only in the stranded gel agglomerates (pH 6.8, sheared) was detected at beginning (1 min) of the intestinal digestion and was not detected in the 2 min digesta sample indicating rapid hydrolysis by proteases and peptidases in pancreatin.

In a recently published study, Boutrou *et al.* (2013) reported peptides released from the digestion of bovine milk proteins in human jejunum.[39] Analysis of degree of hydrolysis showed that whey proteins were hydrolysed to a limited extent by pepsin, but further hydrolysed by pancreatic and intestinal proteases. The resulting peptides were further sequentially modified both from N- and C-terminal ends by aminopeptidases and carboxypeptidases, respectively. Majority of the β-Lg peptides characterized in the jejunum digesta were found to originate from regions R_{40}-K_{60}, K_{75}-T_{105} and Q_{120}-L_{140}, similar to the data obtained in this *in vitro* digestion model (**Fig. 5**).

α-lactalbumin

A total of 35 peptides originating from α-La were identified in the digested WPI solution and structured gels. In **Fig. 6**, primary amino acid sequence of α-La (123 amino acids, 8 Cys residues forming 4 disulphide linkages in native protein) was overlaid with the pepsin, chymotrypsin and trypsin hydrolysis sites together with the peptides identified. Peptides produced during the gastric and intestinal digestion of WPI solution and structured gels originated from α-La were mainly from two distinct regions of the molecule, *i.e.* 1-60 and 80-123. No peptides originating from region 61-79 of α-La was found in neither WPI solution nor structured gel digested samples. Interestingly, region 61-79 of α-La contained no reported/predicted pepsin, chymotrypsin trypsin cleavage site (**Fig. 6**). The region contained 3 of 8 Cys residues in α-La that may be involved in inter- and intra-molecular disulphide linkages rendering potential hydrolysis sites inaccessible to proteolytic cleavage. From the total 35 peptides originating from α-La identified, 6 and 19 peptides were only found in the digested WPI solution and structured gels, respectively (**Fig. 6**). The remaining 10 peptides were found in both digested WPI solution and structured gels (**Fig. 6** and **Table 2**).

The six peptides originating from α-La found only in the digested WPI solution were in the regions of 35-50 (α-La f37-43 and f37-50) and 95-123 (α-La f97-103, f99-117, 100-117 and f104-121). These peptides were produced during the gastric digestion and were further degraded rapidly in the early phase of the intestinal digestion by proteases and peptidases in pancreatin. In the 19 peptides from α-La which were identified only present in the WPI gel digestion samples (**Fig. 6**), the gel structure was found to influence the peptide patterns. Two α-La peptides (α-La f82-89 and f11-18) were only found in the digested aggregate network (non-sheared, **Fig 1e-f**) and particulate agglomerates (sheared, **Fig. 1g-h**) gels made at pH 4.6, respectively. Peptide α-La f105-123, representing C-terminally located 18 amino acid residues, was produced from WPI gels made at pH 6.8 (non-sheared and sheared, **Fig 1 a-d**). Peptide α-La f10-23 was only detected in the gastric

digesta of the sheared gels at both pHs. Structures of WPI gels did not influence the production of peptide α-La f41-49, as it was detected in all four types of WPI gels.

After the *in vitro* digestion proceeded to the intestinal phase, further 10 peptides were produced from α-La. Five of these peptides (α-La f17-27, f81-89, 81-90, f95-102 and f95-103) were detected in the digesta of all four types of gels, indicating microstructure of the gels had little influence on the release of these peptide from α-La. In addition, these peptides remained intact throughout the duration of intestinal digestion. One peptide, α-La f11-16, was only found in the digesta of the sheared gels at both pHs. On the other hand, peptide α-La f94-103 was only produced from the particulate gels (pH 4.6, non-sheared and sheared; **Fig 1 e-h**) during the early stage of intestinal digestion, and was further hydrolysed as the digestion proceeded. The last three peptides were detected during the early stage of intestinal digestion (1 and 2 min digesta samples) of the gels prepared at pH 6.8, sheared (α-La f94-99) or non-sheared (α-La f9-25 and f41-53). There were also α-La peptides detected during the digestion of WPI gels which were produced during the simulated gastric condition and were further hydrolysed only by proteases and peptidases in pancreatin, namely α-La f9-18, f41-52, f53-60 and f92-103. The origins of these peptides were clearly not influenced by the structure of the gels.

Boutrou *et al.* (2013) reported that the majority of α-La peptides identified, in digesta samples collected in human jejunum, originated from region F^{80}-M^{90} and no peptides were found from N- (1-17) and C-terminals peptides.[39] In contrast, the present study which used an *in vitro* model, clearly showed that the majority of the peptides identified in the digested WPI solution and structured gels originating from α-La, were from the regions 10-60 and 80-123. The difference in digesta peptide patterns between the WPI solution and structured gels may be the result of differences in the initial hydrolysis/primary proteolysis, both in terms of the bonds hydrolysed and rate of hydrolysis, producing a range of large peptides. The large peptides, products of primary proteolysis, were then further modified by secondary proteolysis by the gastric and/or intestinal

enzymes yielding differing small peptides patterns observed for the digested WPI solution and structured gels (**Fig. 5** and **6**). The SDS-PAGE gels (**Fig. 3**) clearly highlighted the differing rates and peptide pattern resulting from WPI digestion between the solution and structured gels and in addition the structured gels made with and without shearing.

Influences of supramolecular structures on peptide release

Assembly and network formation of WPI is influenced by a number of different parameters such as polymer concentration, pH, ionic environment and temperature. Although a substantial amount of research has shown differences in the inter- and intra-molecular interactions of WPI, there is limited information about the exact amino acids which are taking part in both chemical and physical bonding to create higher order supramolecular structures and how these structures might impact on the enzymatic hydrolysis of the protein network. However, the results from the characterisation of peptides in the digesta resulting from the simulated gastric and intestinal digestion of WPI solution and gels show that the protein structures (folded/native or unfolded/denatured molecules as well as networks of aggregates or agglomerates) could have a marked effect on the enzymatic digestion and the peptides released. In addition, the data on the characterisation of digesta (**Fig. 3**-**4**) highlighted that there were differences in the rates of peptide release. This was demonstrated by; a) specific enzymatic hydrolysis of whey proteins and the release of peptide products and, b) production of specific peptides and their further hydrolysis (**Fig. 5-6** and **Table 1-2**).

This work highlighted the influence of protein structure (native vs. denatured/gelled) on enzymatic digestion in simulated gastro-intestinal model systems. The heating of whey protein during the formation of gelled structures, dramatically reduced the resistance of whey proteins to pepsin/gastric digestion. During the commercial manufacture of ingredients, protein preparations undergo a range of heating regimes, *e.g.* pasteurization, concentration and drying. Depending on the severity of heating conditions, proteins could potentially undergo a different extent of heat-induced

467 modifications which can have major consequences for protein digestibility and nutritional quality in
468 terms of bioavailability of essential amino acids. Some of these heat-induced changes could include:
469 a) modification of amino acid side chains by Maillard reaction, ε-amino group of lysine; b) β-
470 elimination reactions to cystine/cystein and phosphor-serine residues leading to cross-link
471 formations and, c) formation/interchange of disulphide linkages. These changes can lead to inter-
472 and intra-molecular cross-linkages and modification of amino acid side chains which can directly
473 impact on the accessibility of peptide bonds for enzymatic cleavage.

474 Gastric digestion of ingested food is a complex process involving both physical
475 disintegration and chemical reactions. In addition to structural considerations, one aspect of protein
476 digestion which could affect the rate of protein digestion is the residence time and transit rate of the
477 food in the human GI tract. This has not been accounted for in the *in vitro* study presented in this
478 work. The gastric emptying rate of solution or gel could have major consequences for the rate of
479 protein hydrolysis, order of peptide production/release from parent protein molecule and delivery of
480 bioactive/health enhancing peptides. The work by Barbe *et al.* (2013) showed that liquid milk goes
481 through the stomach much quicker than rennet gel, resulting in a rapid increase in amino acid
482 concentration.[13] The earlier study by Marciani *et al.* (2001) using MRI showed that increasing
483 viscosity of food affected the intragastric dilution and delayed stomach emptying which
484 subsequently influence the feeling of fullness after food consumption.[40] The work by Juvonen *et al.*
485 (2009) also showed that the gastric emptying as measured by paracetamol absorption was faster
486 after low-viscosity oat bran beverage consumption compared with a high-viscosity drink.[41] As a
487 consequence, viscosity differences in oat beta-glucan in a liquid meal with identical chemical
488 composition strongly influenced not only glucose and insulin responses, but also short-term gut
489 hormone responses. This further supports the findings of this work and implies the importance of
490 food structure in the modulation of postprandial satiety-related physiology. The greater rate of
491 stomach emptying of protein in solution might result not only in a different rate of overall protein

hydrolysis but more importantly quite different peptide pattern(s). The results presented in this study using an *in vitro* system provided valuable insights to how protein structures may affect their digestion. However, clinical studies are required to understand to what extent changes in protein supramolecular structure and subsequent peptide release will impact the physiological response(s).

Conclusions

Heat induced whey protein gels were formed at two different pH values, 6.8 and 4.6, with and without applied shear. The protein gels showed different microstructures due to heat induced unfolding under various physico-chemical conditions, resulting in fine stranded (at pH 6.8) or particulate (at pH 4.6) protein networks. When shear was applied during heating, agglomerates with different pH dependent microstructures within the protein networks were formed. The differences in the gel microstructures resulted in considerable differences in their rheological properties. WPI solution and variously structured gels were subjected to simulated gastric and intestinal digestion in an *in vitro* model system. Native whey proteins in solution, β-Lg and α-La, largely survived simulated gastric digestion but hydrolysis progressed rapidly in simulated intestinal digestion by proteases such as chymotrypsin and trypsin. WPI in structured gels were rapidly hydrolysed in simulated gastric digestion. However, the structure of the gels influenced the pattern of the generated peptides. Further research is needed to determine the precise amino acids in the proteins that are specifically taking part in the formation of intermolecular interactions by analysis of the target peptides formed as a result of enzymatic hydrolysis. Although the present results were obtained using an *in vitro* system, the findings revealed that this type of study can provide insights and directions for the development of food formats for further clinical studies to assess whether the release of biologically active peptide sequences at specific points during digestion may yield a modified physiological response, and thus resulting in desired health outcomes.

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Table 1. Peptides identified originating from β-lactoglobulin detected both in solution and structured whey protein gels during simulated gastric and intestinal digestion. The peptides presence (+) or absence (-) are marked in the sequence of gastric digestion at 0, 10 and 120 min (in black) and subsequent intestinal digestion at 1 ,2,4 and 120 min (in red).

Peptide origin	Peptide Sequence	MW	WPI solution	WPI gel			
				pH 4.6 NS*	pH 4.6 S*	pH 6.8 NS*	pH 6.8 S*
β-LG f01-11	LIVTQTMKGLD	1217.7	+++++--	+++++--	+++++--	+++++--	+++----
β-LG f02-11	IVTQTMKGLD	1104.6	-+-----	---++--	--+----	-+-----	-------
β-LG f09-20	GLDIQKVAGTWY	1349.7	-++++--	---+---	----+--	-------	-------
β-LG f10-18	RELKDLKGY	1120.6	-+-----	-------	-------	-++----	-------
β-LG f10-19	LDIQKVAGTW	1129.6	-++++--	-++++--	+++++--	+++++--	+++++-
β-LG f32-41	LDAQSAPLRV	1068.6	+++----	---+---	++-----	--+----	--+----
β-LG f32-42	LDAQSAPLRVY	1231.7	-++----	--+----	--+----	-------	-------
β-LG f41-57	VYVEELKPTPEGDLEIL	1943.0	-++++++	-----++	---+++	------+	-------
β-LG f42-54	YVEELKPTPEGDL	1488.7	+++++++	-++++++	+++++++	+++++++	+++++++
β-LG f42-55	YVEELKPTPEGDLE	1617.8	+++++++	-++++++	+++++++	+++++++	+++++++
β-LG f42-57	YVEELKPTPEGDLEIL	1843.9	+++++++	-++++++	++-++++	---+++	+--++++
β-LG f42-60	YVEELKPTPEGDLEILLQK	2213.2	---++-	-----+-	----++-	-------	----++-
β-LG f43-54	VEELKPTPEGDL	1325.7	+++++++	+++++++	+++++++	+++++++	+++----
β-LG f43-55	VEELKPTPEGDLE	1454.7	+++++++	+++++++	+++++++	+++----	+-------
β-LG f43-57	VEELKPTPEGDLEIL	1680.9	+++++++	+++++++	+++++++	------+	+++++++
β-LG f43-60	VEELKPTPEGDLEILLQK	2050.1	---++-	----++-	-----+-	-------	-------
β-LG f45-54	ELKPTPEGDL	1097.6	+++----	+++----	+++----	+++----	+++----
β-LG f46-54	LKPTPEGDL	968.5	+++----	+++----	+++----	+++----	+++----
β-LG f71-82	IIAEKTKIPAVF	1328.8	-++----	-+-----	-------	-------	---+----
β-LG f74-82	EKTKIPAVF	1031.6	+++++--	+++++--	-++++--	+++----	+++----
β-LG f82-93	KIDALNENKVL	1255.7	---+++-	---+++-	-----+-	-------	---+++-
β-LG f82-95	KIDALNENKVLVL	1467.9	-++----	-++----	-++----	-------	-++----
β-LG f94-104	VLDTDYKKYLL	1369.8	-++----	+++----	-+-----	-------	+-------
β-LG f96-104	DTDYKKYLL	1157.6	+++++--	+++++--	+++----	+++----	+++----
β-LG f96-105	DTDYKKYLLF	1304.7	+++++--	+++++--	+++----	+++----	+++----
β-LG f123-138	VRTPEVDDEALEKFDK	1889.9	---++-	---++-	---++-	---+---	---++-
β-LG f125-138	TPEVDDEALEKFDK	1634.8	---++++	---+++	---+++	+++++++	+---+++
β-LG f132-146	ALEKFDKALKALPMH	1711.0	+++----	+++----	---+---	-++----	-++----
β-LG f132-149	ALEKFDKALKALPMHIRL	2093.2	+++++++	+++----	++++---	+++----	+++----
β-LG f133-149	LEKFDKALKALPMHIRL	2022.2	+++----	+++----	+++----	+++----	+++----

β-LG f134-145	EKFDKALKALPM	1389.8	+++----	+++----	-------	-++----	-++----
β-LG f134-146	EKFDKALKALPMH	1526.8	++++---	++++---	++++---	++++---	-++----
β-LG f134-149	EKFDKALKALPMHIRL	1909.1	-++++++	+++++++	+++++++	+++++++	+++++++
β-LG f135-149	KFDKALKALPMHIRL	1780.1	-++----	+++----	+++----	+++----	+++----
β-LG f137-149	DKALKALPMHIRL	1504.9	+++----	+++----	+++----	+++----	+++----
β-LG f140-149	LKALPMHIRL	1190.7	+++----	+++----	+++----	+++----	+++----
β-LG f149-162	LSFNPTQLEEQCHI	1656.8	---++-	---++-	-------	-------	-------
β-LG f150-162	SFNPTQLEEQCHI	1544.7	---+++-	-------	-------	---++--	---+++-

*NS – gel prepared without shearing; S – shear was applied during gelation.

Table 2. Peptides identified originating from α-lactalbumin detected both in WPI solution and various structured gels of whey proteins during simulated gastric and intestinal digestion. The peptides presence (+) or absence (-) are marked in the sequence of gastric digestion at 0, 10 and 120 min (in black) and subsequent intestinal digestion at 1 ,2,4 and 120 min (in red).

Peptide origin	Peptide Sequence	MW	WPI solution	WPI gel			
				pH 4.6 NS*	pH 4.6 S*	pH 6.8 NS*	pH 6.8 S*
α-LA f 10-18	RELKDLKGY	1120.6	-++++++	+++++---	+++++--	+++++++	+++++++
α-LA f 10-20	RELKDLKGYGG	1234.7	-+------	+++++++	-------	-+------	-------
α-LA f 12-18	LKDLKGY	835.5	-+++---	-+++---	-------	++++---	++++---
α-LA f 19-27	GGVSLPEWV	942.5	---+-++	+++++++	-----++	+++++--	+------
α-LA f 32-40	HTSGYDTQA	978.4	-------	-------	-++++---	-++++++-	+++----
α-LA f 53-59	FQINNKI	875.5	---+++	-------	-+------	-------	-------
α-LA f 80-89	FLDDDLTDDI	1180.5	---+++	+++++++	---+++	---++--	---+++
α-LA f 80-90	FLDDDLTDDIM	1311.6	-----++	---+++	---+++	----+--	---+++
α-LA f 82-90	DDDLTDDIM	1051.4	-----++	---+++	---+++	----+--	---+++
α-LA f 104-123	WLAHKALCSEKLDQWLCEKL	2394.2	-++++---	+++++---	+++----	-++++---	+++++---

*NS – gel prepared without shearing; S – shear was applied during gelation.

Fig. 1. Protein unfolding, network formation and aggregation resulting in different structures of gels formed at pH 4.6 and 6.8 with or without shear. Images a, c, e and g are 250 × 250 µm, and images b, d, f and h are 50 × 50 µm.

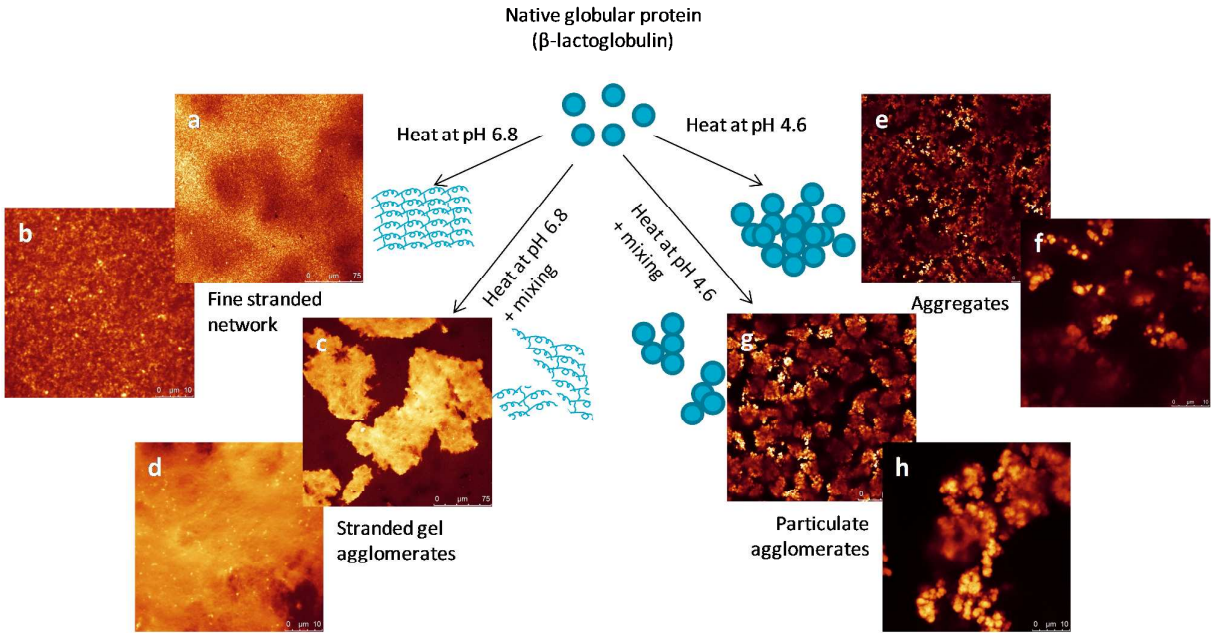

Fig. 2. Rheological properties of heat set 10% w/v whey protein networks at 20 °C. The gels were prepared in the rheometer by heating the protein solutions at pH 4.6 or 6.8 to 90 °C at a heating rate of 5 °C/min. The frequency sweeps were carried out after the gels were cooled down to 20 °C. Gels prepared at (A) pH 4.6; and (B) pH 6.8. Gels prepared without shear: *G*' (▲), *G*'' (△), tan δ (●); or with shear: *G*' (▼), *G*'' (▽), tan δ (●).

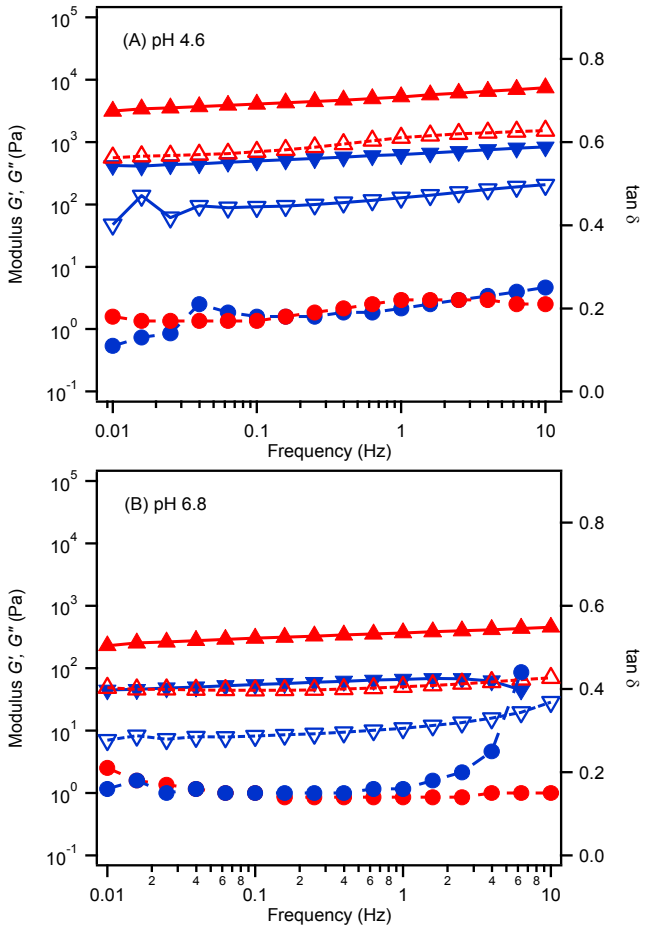

Fig. 3. SDS-PAGE of whey protein in solution and structured gels prepared at pH 6.8 or 4.6 with or without shear, digested samples drawn at different time during simulated gastric (Lanes/time) and intestinal (Lane/time) digestion. Lanes 1 and 13: molecular weight marks; lanes 2: undigested whey protein solution or gels; lanes 3-7: digested in simulated gastric fluid, samples taken at 0.5, 1, 4, 10 and 120 min digestion; lanes 8-12: proteins digested in simulated gastric fluid for 120 min followed by simulated intestinal digestion at 0.5, 2, 4, 15 and 120 min.

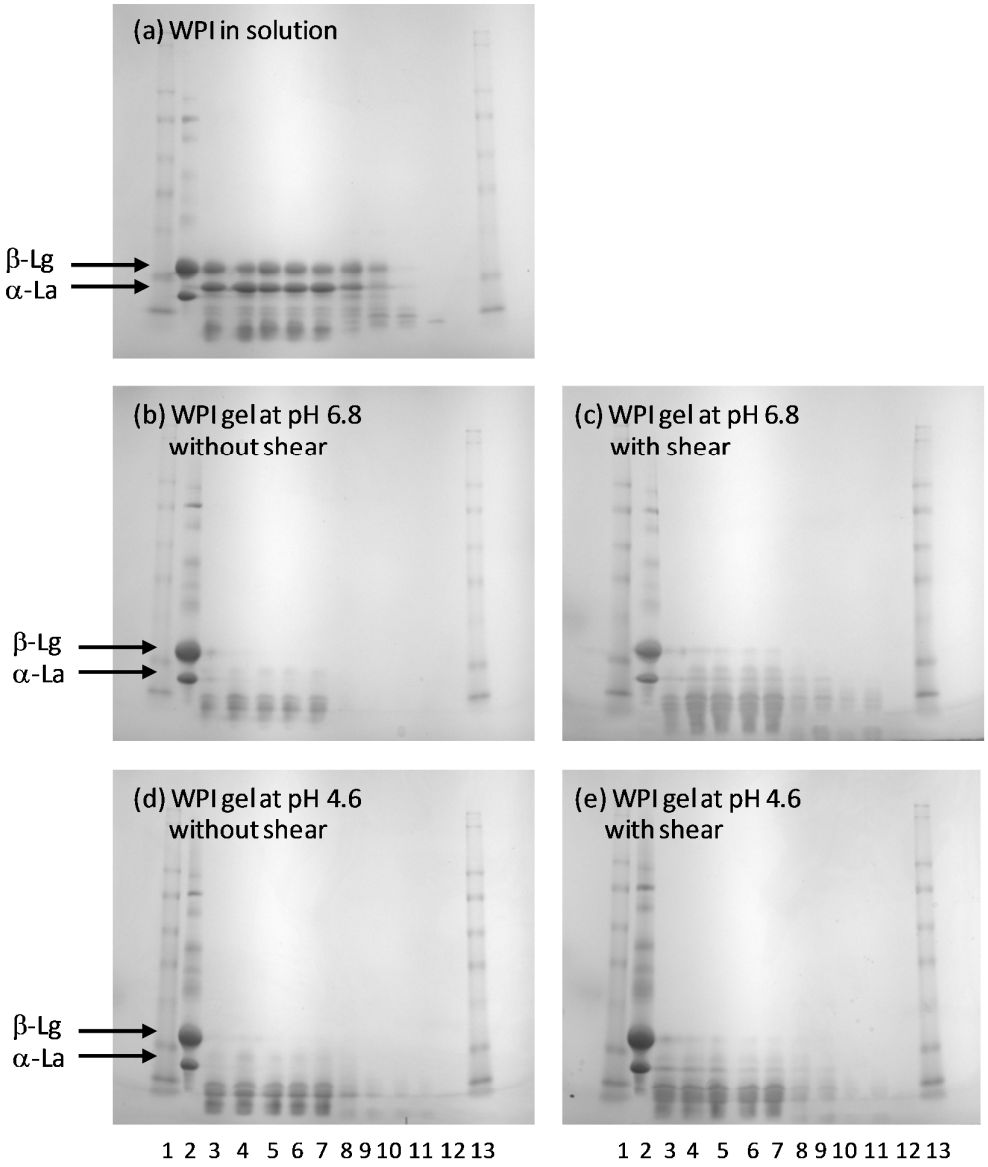

Fig. 4. RP-HPLC chromatograms of digested whey protein in solution and gel samples, taken at: (a) 10 min into the simulated gastric digestion; and (b) 4 min into simulated intestinal digestion following 120 min gastric digestion.

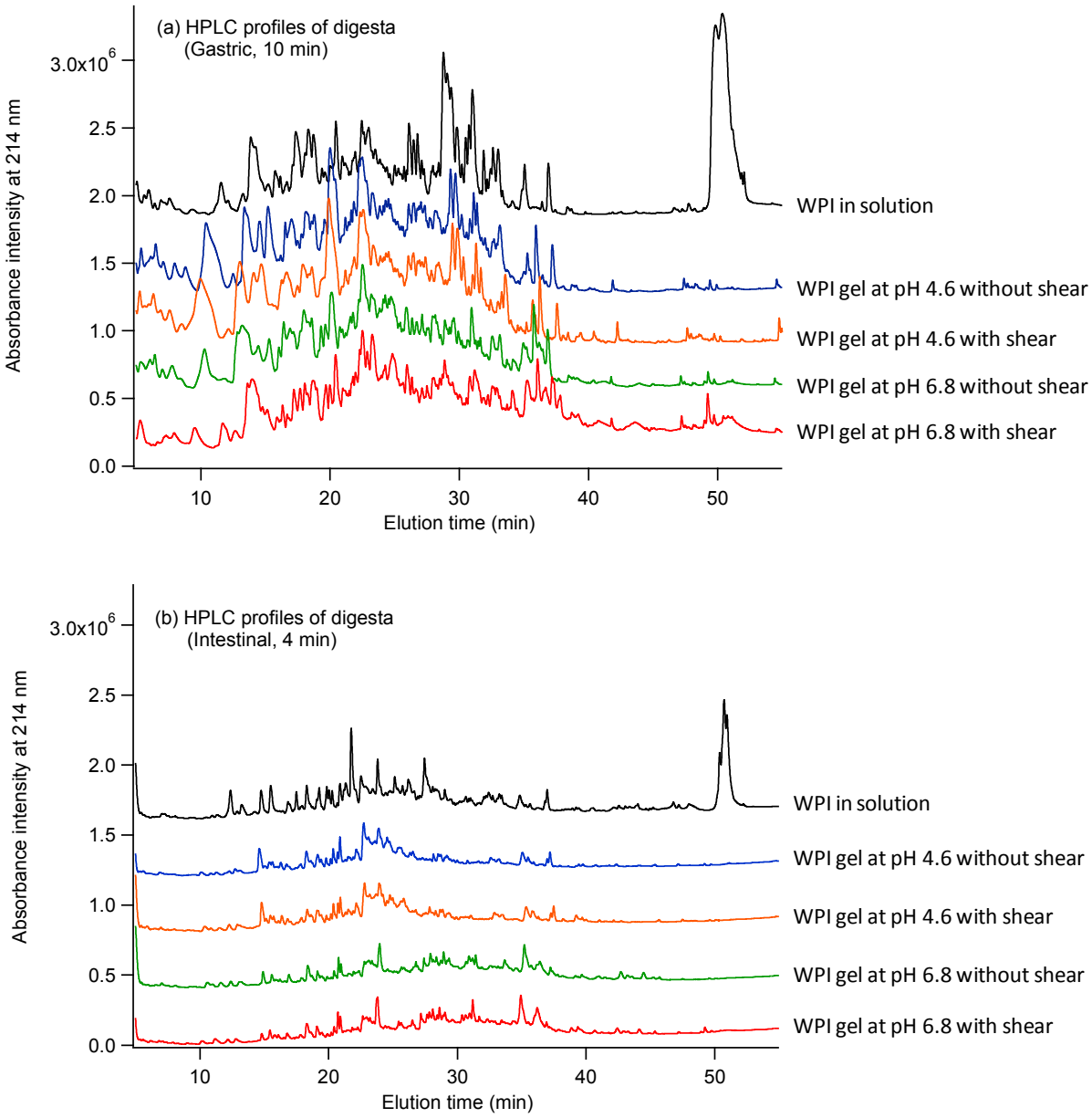

Fig. 5. Peptides originating from β-lactoglobulin produced during simulated gastric and intestinal digestion of whey proteins in solution and variously structured gels. Hydrolysis sites, as predicted by PeptideCutter – ExPASy Bioinformatics Resources Portal (Swiss Institute of Bioinformatics, http://web.expasy.org/peptide_cutter/), of pepsin (↑), chymotrypsin (◆) and trypsin (↓) marked on the primary amino acid sequence of β-lactoglobulin. Blue lines indicate peptides present in WPI solution digesta. Red lines indicate peptides present in the digesta of all four structured gel samples. Black lines represent peptides found in both WPI solution and structured gels.

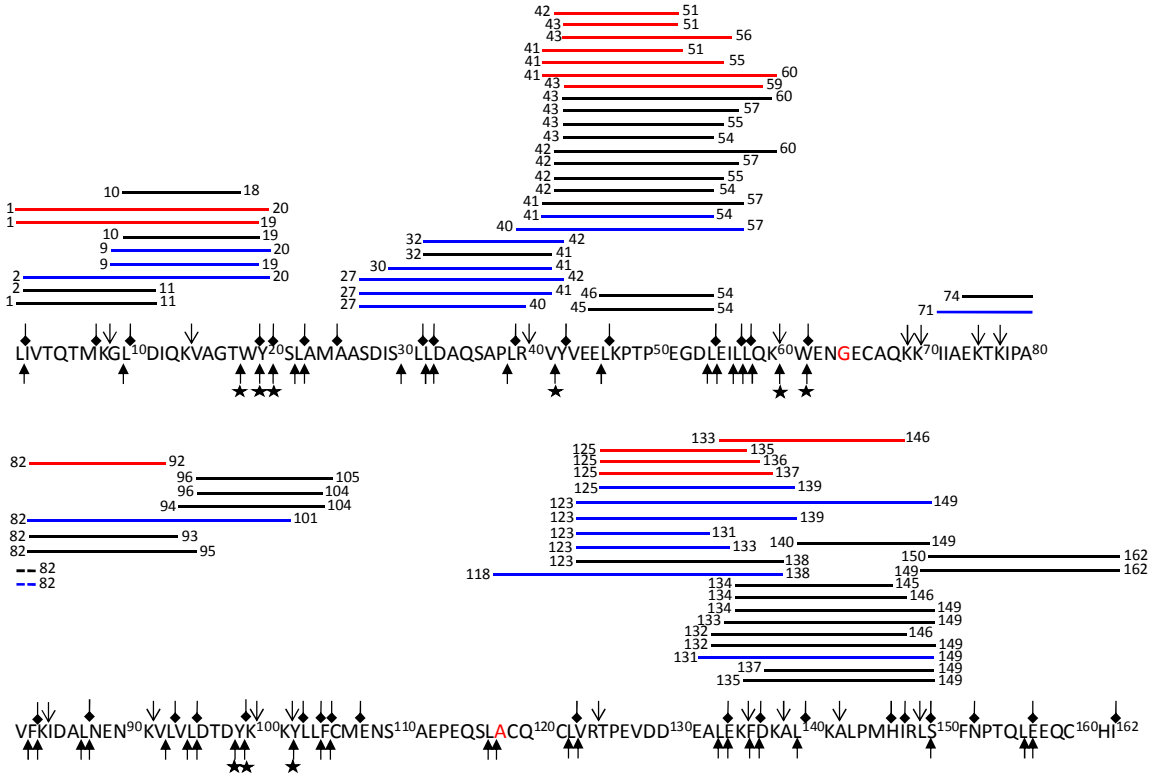

Fig 6. Peptides originating from α-lactoalbumin produced during simulated gastric and intestinal digestion of whey proteins in solution and variously structured gels. Hydrolysis sites, as predicted by PeptideCutter – ExPASy Bioinformatics Resources Portal (Swiss Institute of Bioinformatics, http://web.expasy.org/peptide_cutter/), of pepsin (↑), chymotrypsin (◆) and trypsin (↓) marked on the primary amino acid sequence of α-lactoglobulin. Blue lines indicate peptides present in WPI solution digesta. Red lines indicate peptides present in the digesta of all four structured gel samples. Black lines represent peptides found in both WPI solution and structured gels.