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Digestive diversity and kinetic intrigue among heated and unheated β-lactoglobulin species

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Food processing often alters the structure of proteins, and proteins are deliberately denatured and aggregated to improve technological functionality in many cases. However, the digestive consequences of processing-induced alterations to protein structure have only recently been studied. Here we explored the process-structure-digestibility relationship in the context of heat-processing effects on the structure and gastric digestibility of bovine whey protein β-lactoglobulin (β-lg). Heating β-lg produces an array of non-native monomers, dimers and aggregates, and we have characterised these with reverse-phase high performance liquid chromatography (RP-HPLC) as a complement to our earlier work using polyacrylamide gel electrophoresis (PAGE) techniques. Using a combination of SDS-PAGE and RP-HPLC we have identified pepsin-resistant dimers and peptides that appear early in digestion. In an unexpected finding, native β-lg underwent complete hydrolysis during prolonged incubation (48 h) with pepsin. Two phases of hydrolysis were identified, and the transition between phases appears to result from alterations to the secondary structure of β-lg at 3-4 h, as measured with circular dichroism spectroscopy. This work has unpacked some of the complexities of the processing-structure-digestibility relationship in a highly simplified system; further work is needed to explore the implications of these findings for food processors, regulatory authorities and consumers.

Introduction

In the last five years there has been a surge of interest in the biological processes involved in consuming and digesting foods. This is driven in part by the recognition that food structure influences how a food breaks down during biological processing, and thereby affects nutrient uptake and physiological outcomes. The broader context is that the health impacts of food can potentially be modulated by selecting food components and processing conditions to achieve a desired structure[1-3].

However, a systematic approach to realising this potential requires a mechanistic understanding of the processing-structure-digestibility relationship. Whilst there is ample food science literature on the processing-structure link, and plentiful biochemical literature on the molecular mechanisms of digestive enzymes, few studies have integrated all three elements. Here we demonstrate the integrated processing-structure-digestibility approach by studying the gastric digestibility of bovine β-lactoglobulin (β-lg) after various heat treatments.

Heating is one of the most common unit operations in the food industry, and the purpose of heating may be to ensure microbiological safety or to alter food structure or chemistry in desirable ways. Proteins from mesophilic organisms, the sources of most proteinaceous foods, are very responsive to temperature because their secondary and tertiary structure elements involve extensive hydrogen bonding and hydrophobic interactions. Once heat-denatured, proteins are often susceptible to interactions with other proteins, which may be of a noncovalent and/or covalent nature, and the structure of aggregated proteins has a strong influence on their susceptibility to proteolytic enzymes, including digestive enzymes in the body[4, 5].

Native β-lg is generally considered to be stable under gastric conditions[6-9], although it must be pointed out that gastric conditions vary dramatically between individuals, meal types and fed/fasted states.[10, 11] For example, the enzyme:substrate ratio may vary over nearly four orders of magnitude[12], phospholipid components can affect protein digestion[13], and gastric pH for a given individual ranges from 1.3 in the fasted state to as high as 5.8 (or even higher for an infant) immediately after a meal[14]. The native tertiary structure of β-lg is stabilised by two disulphide bonds as well as by electrostatic interactions and hydrogen bonds. The hydrophobic residues at which pepsin cleaves are within the protected interior of the protein. The disruption of any of these stabilising forces by alteration of the physical and/or chemical environment leads to

an increase in susceptibility to pepsin digestion, e.g. in alcoholic solvents[9, 15], in the presence of a reducing agent[16], at elevated temperature[16], or at an oil/water interface[4]. Peptic digestion of β-lg is inhibited by the presence of certain ligands,[17, 18] and the presence of glucose during heating reduces susceptibility to pepsin[19].

This article builds on our previous work[5], which examined the complex suite of denatured and aggregated species created by heating β-lg, and revealed both highly-digestible and digestion-resistant species. Here we examine the heat-induced structure modifications to β-lg, augment earlier electrophoretic analysis with reverse-phase chromatography and reveal a new twist in the story of how native β-lg resists peptic hydrolysis.

Experimental

Materials

β-Lactoglobulin from bovine milk was either supplied by Sigma-Aldrich (> 90% pure) or isolated in-house from whey protein isolate (Fonterra Co-operative, Auckland, New Zealand) using salt-precipitation followed by dialysis and freeze-drying[20], and was 97% pure. In both cases the protein was a mixture of genetic variants A and B. Porcine pepsin (800 to 2500 units/mg protein) was supplied by Sigma-Aldrich (St. Louis, MO), as were other chemicals (analytical grade) unless otherwise specified.

Sample preparation, heating and *in vitro* digestion

β-Lg was dissolved at 5 mg.mL^{-1} in Milli-Q water at pH 7.4. Heating experiments used the same protocols as in earlier work[5], except that the water bath temperature was in some cases 78 ± 0.1 °C, and for some experiments β-lg was heated in buffer (50 mM Na$_2$HPO$_4$, 50 mM NaCl). Samples were heated in glass tubes for the required times, then cooled in a water-ice slurry for 5 min.

Simulated gastric fluid for *in vitro* digestion consisted of 3.2 mg.mL^{-1} pepsin in 35 mM NaCl adjusted to pH 1.2 with HCl. SGF and β-lg solutions were pre-heated at 37 °C before mixing together in proportions that gave a pepsin:β-lg ratio of 1:6.

Polyacrylamide gel electrophoresis (PAGE)

Alkaline native PAGE followed the same method as our previous work[5]. Tris-tricine reduced sodium dodecyl sulphate PAGE (SDS-PAGE) was done according to a published method[21]. Bands on PAGE gels were quantified by densitometry, using a Geldoc XR system (Bio-Rad Laboratories, CA). Kinetic data were fitted using nonlinear regression in Sigmaplot 12.5 (SyStat Software Inc).

Circular dichroism (CD) spectroscopy

A Chirascan spectroscope (Applied Photophysics Ltd, UK) was used for CD spectroscopy, with cuvettes of pathlength 10 mm (near UV) or 0.1 mm (far UV). Spectra were collected with 1 nm steps and 0.5 s per point, and 10 replicate spectra were averaged. Spectra were smoothed with Sigmaplot using a 3rd order polynomial function to fit windows of 8 points with Gaussian weighting and automatic outlier rejection.

CD spectra were deconvoluted and compared to spectra in the database of CAPITO[22]. Deconvolutions of spectra beyond 12 h, due to uncertainty in concentration of species yielding CD signals and heterogeneous nature of the sample once pepsin was added, were ambiguous. However, spectral similarity to species present in the CD database, PCDDB[23], was visually apparent.

Reverse-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out using a 1200 series Agilent HPLC and an Agilent automatic injector (Agilent Technologies, Palo Alto, CA), with a diode array detector set at 214 nm. Separation used an Alltech Prevail C18 column with particle size 5 μm and dimensions 4.6 x 150 mm (Grace, Illinois, USA), which was connected to a guard cartridge system and was operated at a flow rate of 0.3 mL.min^{-1} at 20 °C. All samples were filtered through a 0.45 μm syringe filter before injection. The injection volume was 30 μL, and samples were eluted using linear gradients of solvent A (10 % v/v acetonitrile, 90 % v/v H$_2$O with 0.1 % v/v trifluoroacetic acid) and solvent B (90 % v/v acetonitrile, 10 % v/v water with 0.08 % v/v trifluoroacetic acid).

The mobile phase for the first 3 min was 100 % A, then a linear gradient of 100 % A to 55 % A was applied using solvent B over 45 min, then a sharper gradient to 30 % A over the next 5 min. This composition was maintained for 5 min then returned to 100 % A over 5 min. EZ Chrom Elite software (version 3.3.1) was used for data acquisition.

Data analysis

SigmaPlot 12.5 (SyStat Software Inc.) was used for nonlinear regression analysis, and other calculations were done in Microsoft Excel, version 2.26. All uncertainty figures given after equation parameters are standard errors.

Results and Discussion

Heat-induced denaturation and aggregation of β-lg

Heating at 78 °C led to denaturation and aggregation of β-lg, as evidenced by progressive fading of the two native monomer bands (genetic variants A and B) and the appearance of non-native and aggregated species above the monomer bands (Fig. 1). Aggregation was slower than at 90 °C,[5] but the same species appeared in the gel – non-native monomers, dimers, trimers, tetramers, etc.

Using nonlinear regression, native PAGE band intensities were fitted with the integrated general rate law:[24]

$$C = [(n-1)kt + C_0^{1-n}]^{\frac{1}{1-n}} \qquad n \neq 1 \qquad (1)$$

In Eq. 1 C is concentration (raw intensity in this case), n is the order with respect to time, k is a rate constant, t is time and C_0 is the initial concentration.
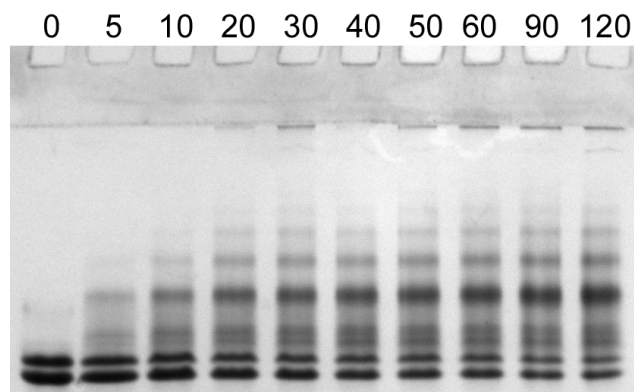

Fig. 1 Alkaline native PAGE gel showing β-lg denaturation and aggregation during heating at pH 7.4 and 78 °C for 0-120 min. Heating times (min) are indicated at the top of the gel.

When Eq. 1 was fitted to PAGE band intensities, influence diagnostics indicated that the first data points at $t = 0$ had a very strong influence on parameters in more than half of the datasets fitted, for example Cook's distance >700 and DFFITS >150 were not uncommon. Removing data points at $t = 0$ from the regression analysis eliminated the problem, and the influence of these data points was confirmed by shifts in parameters, particularly for datasets corresponding to β-lg heated at 90 °C in buffer.

Fitted parameters are summarised in Table 1. Previous reports of heat-induced β-lg denaturation in milk have usually produced n values between 1 and 2, but higher values have also been reported[25]. No previous reports have analysed data without transforming or normalising, and different statistical procedures may contribute to generally higher n values here.
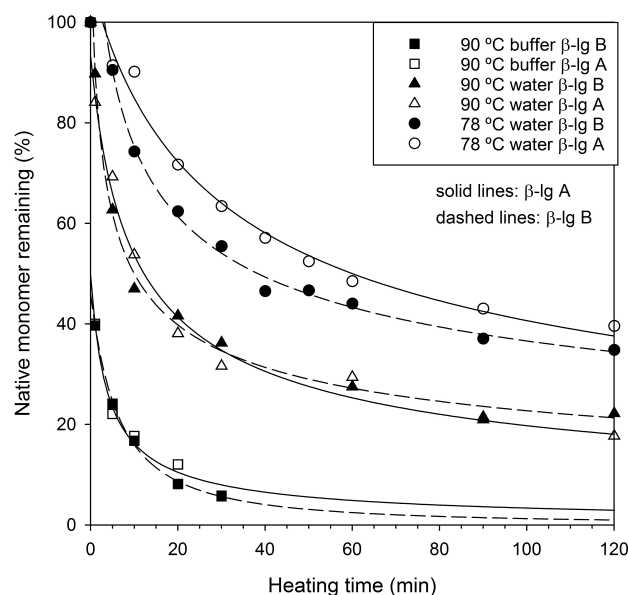

Fig. 2 Kinetics of native β-lg disappearance during heating of 5 mg.mL^{-1} protein solutions at pH 7.4 under different conditions. 'Buffer' contains 0.05 M sodium phosphate and 0.05 M NaCl. Lines show the fit with Eq. 1.

When β-lg is heated in water or buffer, increasing protein concentration decreases n, to the extent that the loss of native β-lg during heating in water or buffer follows first-order kinetics ($n = 1$) at high concentration[26]. The protein concentration used in our experiments (5 mg.mL^{-1}) was relatively low compared to other studies, and the relatively high n values here are consistent with these concentration effects.

Values of k were an order of magnitude higher for heating in buffer, compared to heating in water. This may reflect a weakening of salt bridges resulting from increased shielding at higher ionic strength, which destabilises the native structure of β-lg.

Table 1 Kinetic parameters (±SE) for Eq. 1 fitted to native PAGE band intensities for β-lg heated under various conditions. Figures in brackets are standard errors.

temperature	variant	solution	n (-)	k (min^{-1} ×10^3)	C_0 (%)[a]	RSS[b]
90	A[c]	buffer	1.68 (0.14)	68 (6)	49 (8)	0.007
90	B[c]	buffer	2.3 (0.6)	41 (16)	45.9 (1.5)	0.072
90	A[d]	buffer	1.91 (0.19)	68 (6)	46 (7)	0.007
90	B[d]	buffer	2.2 (0.6)	54 (18)	46.5 (2.0)	0.073
90	A	water	3.8 (0.3)	0.8 (0.3)	93 (5)	0.129
90	B	water	2.9 (0.3)	1.3 (0.7)	112 (9)	0.492
78	A	water	4.0 (0.3)	7.6 (0.5)	108 (7)	0.006
78	B	water	3.1 (0.5)	3.0 (0.9)	138 (24)	0.039

a: fitted C_0 values are expressed as a percentage of the original native protein level, i.e. they are normalised post-regression.

b: RSS, residual sum of squares.

c: first replicate.

d: second replicate.

The k values for heating in water at 78 °C were higher than those for heating in water at 90 °C, which is counterintuitive. However this can be reconciled mathematically by considering that for each variant, fitted C_0 values were substantially higher and n values were slightly higher for the 78 °C data. This is not a satisfying explanation from a mechanistic point of view, but it reflects the fact that Eq. 1 is inherently over-parameterised for the amount of data available here, resulting in some redundancy among parameters. This is supported by the observation that standard errors were large for k, and variance inflation factors were generally high. Fitted C_0 values were greater than 100 % in some cases, though not significantly different from 100 % and probably result from a slight delay before the temperature inside sample tubes reached the nominal heating temperature.

The species created by heating β-lg were examined with RP-HPLC (Fig. 3). Increasing heating time at 90 °C led to a decrease in the two native β-lg peaks (genetic variants A and B), and the appearance of species eluting earlier and later than native β-lg. The amounts of non-native species were approximately constant between 60 and 120 min, whereas the native peaks continued to decrease with more than 60 min heating. This may be accounted for by considering that large aggregates will be filtered out prior to sample injection, and will not appear on chromatograms. However these large aggregates are rapidly digested into species small enough to pass through filters[5], so digestion products will detected by chromatography.
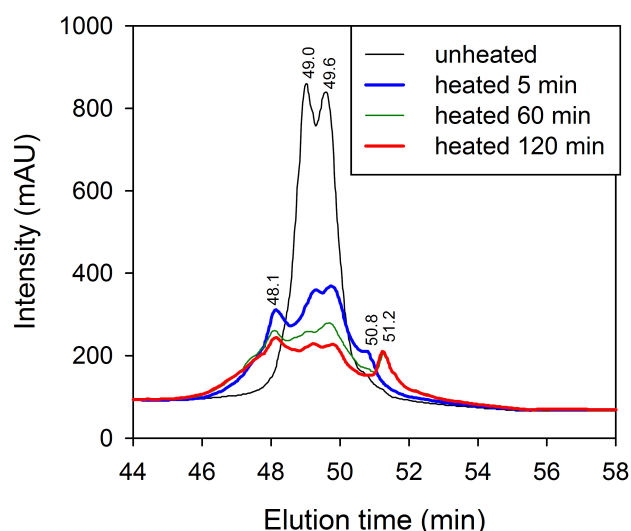

Fig. 3 RP-HPLC chromatograms of β-lg heated for 0-120 min at 90 °C.

Peptic hydrolysis of heated β-lg

In our earlier work[5] we examined the digestion of heated β-lg using a range of PAGE techniques, and reported that heating creates a suite of non-native monomeric and aggregated species. Earlier work used relatively low pepsin:protein ratios and long digestion times by physiological standards,[10, 11] so that subtle changes could be resolved clearly. A similar approach has been taken here, and it should be noted that that phenomena observed in this study cannot be directly extrapolated to infer events *in vivo*.

An example of these results is reproduced in Fig. 4, which shows highly digestible heated species as well as pepsin-resistant species: dimers, intermediate molecular weight species and non-native monomers. This range of species is present at heating times from 5 to 120 min, and the distribution of protein among these various species is determined by the heating time[5].

In the present work we analysed the digestion products of heated β-lg using RP-HPLC, which has higher precision than PAGE and is more sensitive to small peptides. Fig. 5 shows chromatograms of β-lg heated for 5 min or 120 min and digested for 0, 1 or 120 min.

Chromatograms show that the early digestion products are generally more hydrophobic and/or larger than advanced digestion products, as shown by the progressive appearance of more early-eluting peaks and disappearance of late-eluting peaks. The SDS-PAGE gel in Fig. 4 and the RP-HPLC chromatograms in Fig. 5 tell a similar story, and it is possible to match up PAGE bands with chromatogram peaks in some cases. Peaks at 49.7 min and 49.2 or 49.3 min contain native β-lg monomers, but they decrease substantially with 1 min digestion, which suggests that some highly-digestible non-native monomers are also present in these peaks.

The peak at 48.1 min disappears with 1 min of digestion in β-lg heated for either 5 or 120 min. In β-lg heated for 5 min (Fig. 5A), digestion for 1 min leads to the appearance of a peak at 47.3 min, which is largely intact after 120 min, having shifted slightly to 47.5 min. A similar phenomenon occurs with β-lg heated for 120 min, though peaks are much smaller and occur at 47.2 and 47.6 min. The level and persistence of peaks at 47.2 – 47.6 min are consistent with the behaviour of highly digestion-resistant dimers in SDS-PAGE gels. However, the fact that the appearance of these peaks corresponds with the disappearance of the 48.1 min peak suggests that digestion-resistant dimers are the products of early hydrolysis, and may be part of larger species at first.

There is no peak at 50.8 min in unheated β-lg (Fig. 3), but this peak appears when β-lg is heated for 5 min, and it progressively diminishes with longer digestion time. This is consistent with the appearance and loss of moderately digestion-resistant intermediate molecular weight species (Fig. 4A), which consist of a β-lg monomer disulphide-bonded to a peptide.[5]

In β-lg heated for 120 min, the peak at 51.2 min appears with heating then disappears within 10 min of digestion (data not shown). This mirrors the behaviour of some oligomers and aggregates seen at the top of the stacking gel in SDS-PAGE (Fig. 4B).

RP-HPLC reveals much more detail than SDS-PAGE about the peptides produced by hydrolysis of larger species. Peptides eluting at 31 - 45 min were highly transient, and had mostly disappeared within 30 min (data not shown). Peptides eluting earlier can be divided into two categories: those that accumulated with increasing digestion time and those that stayed at approximately constant levels. In β-lg heated 5 min (Fig. 5A), examples of accumulating peptides are those eluting at 14.3, 17.1, 20.4, 21.7, 22.7 and 24.9 min. Peptides that were present after 1 min digestion and did not accumulate further

include those at 21.0, 28.1 and 30.2 min. Heating for 120 min produced much the same pattern: accumulating peptides at 14.0, 17.1, 20.3, 21.6, 22.6 and 24.8 min and approximately constant amounts of peptides eluting at 20.8, 22.2, 28.0 and 30.1 min.

This 'triangulation' of RP-HPLC peak identities using SDS-PAGE makes possible the targeted collection of heating and digestion products from RP-HPLC effluent for further structural analysis. RP-HPLC has revealed a number of digestion-resistant peptides that were not visible in PAGE gels, and has led us to suggest that highly digestion-resistant dimers are initially part of larger 'parent' species, from which they are released in the early stages of hydrolysis.
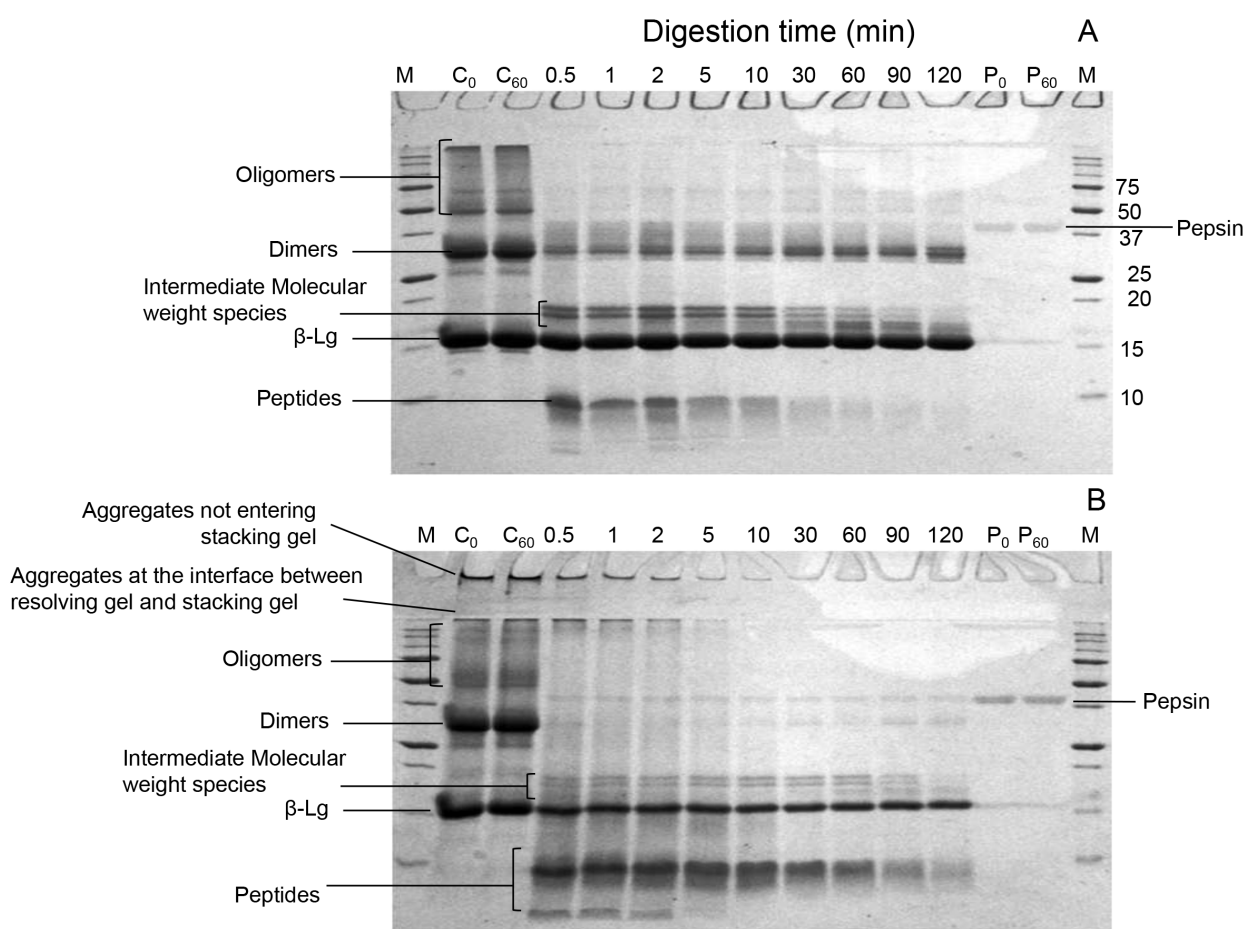

Fig. 4 Nonreduced SDS-PAGE analysis of *in vitro* gastric digestion of β-lg heated at 90°C for (A) 5 min and (B) 120 min. Pepsin:β-lg ratio 1:6. Lane M is the molecular weight markers. C_0 and C_{60} represent preheated β-lg in SGF without pepsin at $t = 0$ and 60 min. The number at the top of each lane represents the time of digestion (min). P_0 and P_{60} represent pepsin controls at $t = 0$ and 60 min. Reprinted from Peram et al.[5] with permission from Elsevier.
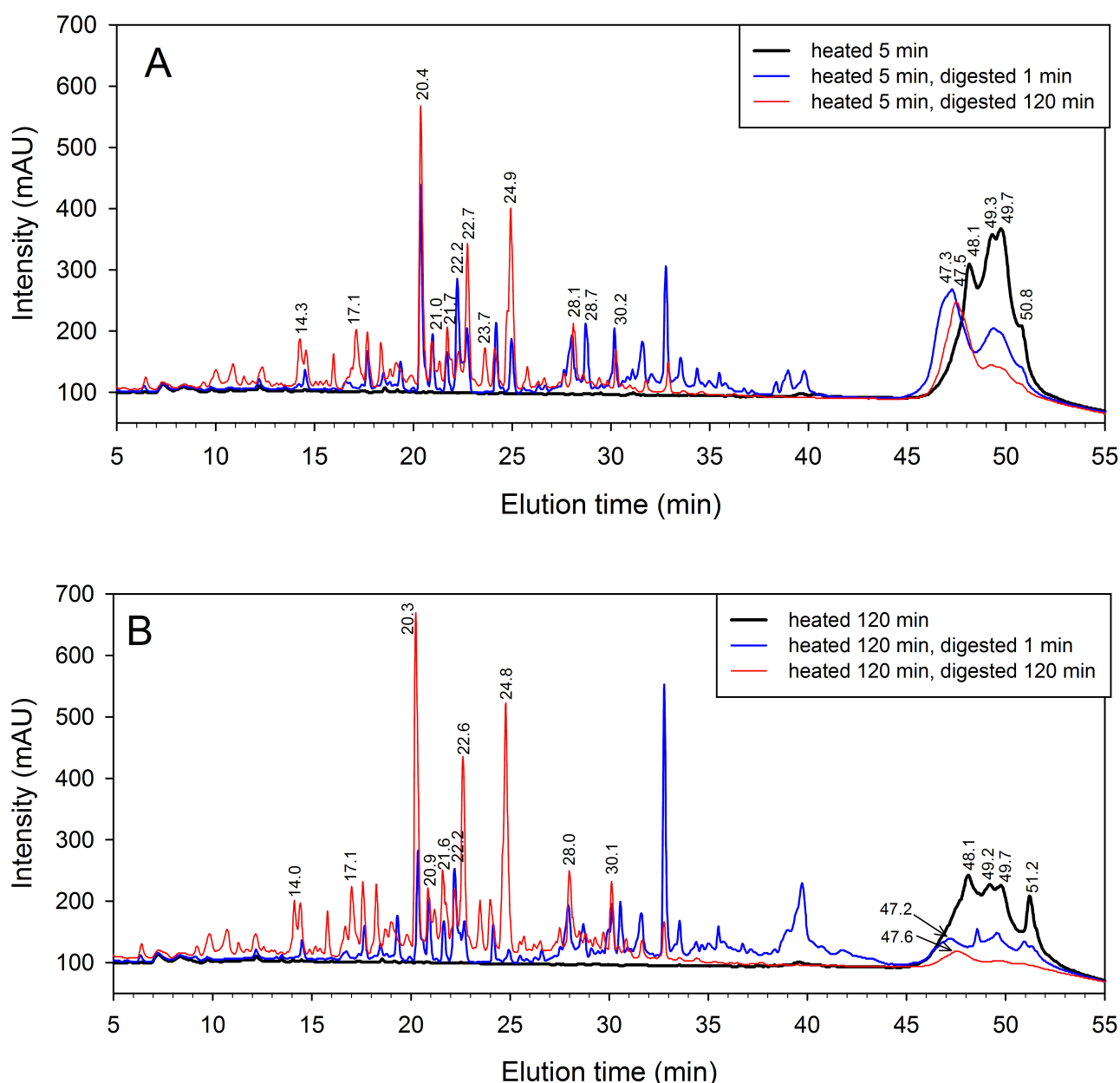
Fig. 5 RP-HPLC chromatograms of heated and digested β-lg. Heating times were 5 min at 90 ˚C (A) or 120 min at 90 ˚C (B). Peaks labelled with elution times are discussed in the text.

Peptic hydrolysis of native β-lg

It has been reported that native β-lg is resistant to pepsinolysis at short times and low pepsin:β-lg ratios [6, 16]. However biological pepsin:protein ratios are thought to span a range approaching four orders of magnitude, and this is reflected in a wide span of experimental conditions for in vitro digestion experiments.[12] Here and in previous work[5] we used a pepsin:β-lg ratio close to the middle of the reported *in vivo* range. In the present work we extended the time span of *in vitro* digestion up to 48 h to examine what, if any, changes to β-lg structural integrity occurred during incubation with pepsin. The

experiment was carried out with both Sigma β-lg and β-lg purified in-house from whey protein isolate using a salt precipitation method[20]. Fig. 6 shows the SDS-PAGE result for the β-lg purified in-house, and the corresponding quantified and normalised band intensities are shown in Fig. 7.

Densitometry results revealed that digestion over time followed two distinct kinetic regimes: initially slow digestion up to a threshold of ~15 % of native β-lg, and subsequent rapid digestion of the remainder. In both regimes data were fitted well by a simple logarithmic decay equation:

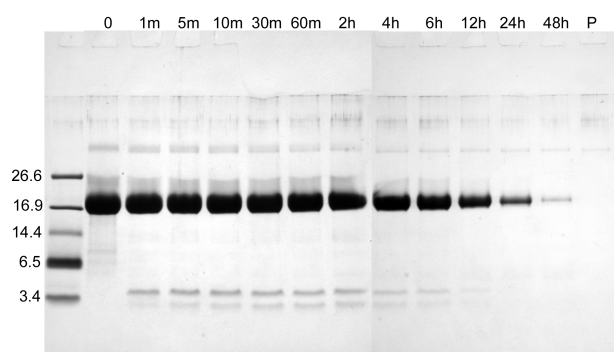$$C = C_1 - m \ln t \qquad (2)$$

Fig. 6 Tris-tricine reduced SDS-PAGE gel showing the digestion of native β-lg during incubation in SGF. Incubation times are indicated above lanes (m, minutes; h, hours); P, pepsin-only control. Numbers on the left indicate molecular weights in kDa.
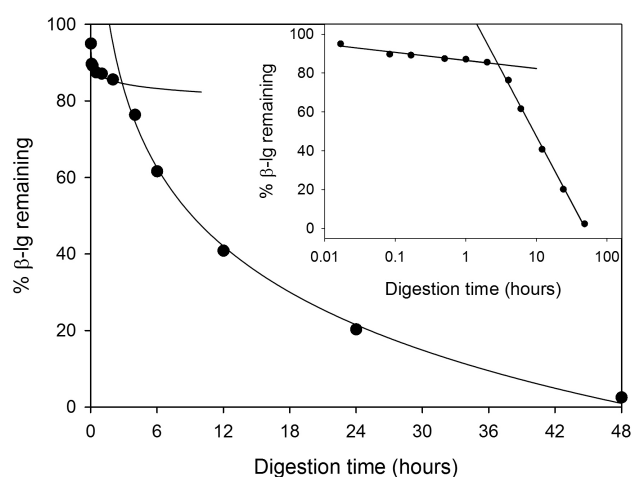

Fig. 7 Two-phase digestion of native β-lg in SGF at enzyme:substrate of 1:6. Lines are fitted with Eq. 2, and the inset shows the same data on a logarithmic x-axis.

The transition between regimes, i.e. the intersection of fit lines in Fig. 7, was at 4.2 h for the Sigma β-lg and 5.5 h for in-house purified β-lg. We hypothesise first that the β-lg monomer-dimer equilibrium could affect pepsinolysis, since cleavage sites would be less accessible in the dimer. β-Lg is mostly dimeric in SGF at ionic strength of 28 mM,[27] and to test this hypothesis β-lg was digested in SGF at reduced ionic strength of 7 mM, at which the monomer will predominate.[27] Under these conditions, digestion again followed two regimes, and the transition occurred earlier at 2.9 h, which provides tentative support for our hypothesis.

With limited replication it is difficult to judge whether differences between the three treatments are significant or meaningful. However, the fact that digestion data followed the same pattern in all cases indicates that the two-regime nature of native β-lg digestion is consistent.

The evolution of protein tertiary structure during digestion was investigated using circular dichroism (CD) spectroscopy. Solutions of native β-lg from Sigma were incubated at 37 °C with pepsin:β-lg of 1:6, and CD spectra in the near-UV (NUV) and far-UV (FUV) regions were recorded at various times.

Control FUV and NUV spectra for pepsin in SGF without β-lg were also recorded (see Electronic Supplementary Information), and these were subtracted from β-lg spectra in all cases. Pepsin spectra had a broad, shallow trough at 200 nm, but pepsin made a negligible contribution at other wavelengths. Near-UV CD spectra (Fig. 8) had characteristic troughs at 293 nm and 286 nm, which are attributed to tryptophan.[28] The depth of troughs diminished progressively with increasing time, as shown in Fig. 8. In Fig. 9, the trough ellipticity at each time subtracted from the final ellipticity at 48 h, $\theta_t - \theta_{48}$, is plotted against digestion time and fitted with Eq 3:
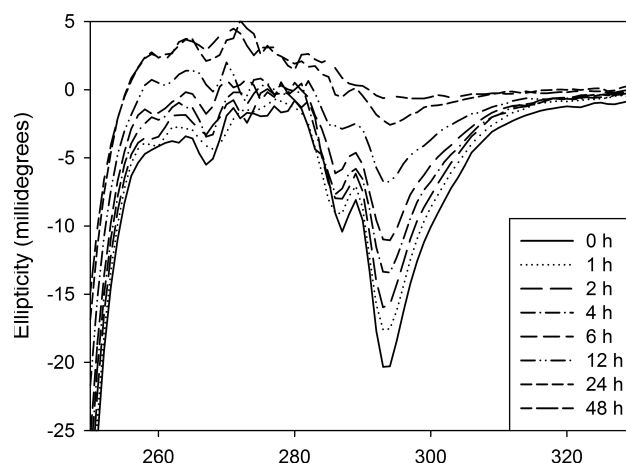
$$\theta_{48} - \theta_t = A exp(Bt) \quad (3)$$

Fig. 8 Near-UV CD spectra of native β-lg during digestion with pepsin (ratio 1:6) at 37 °C.

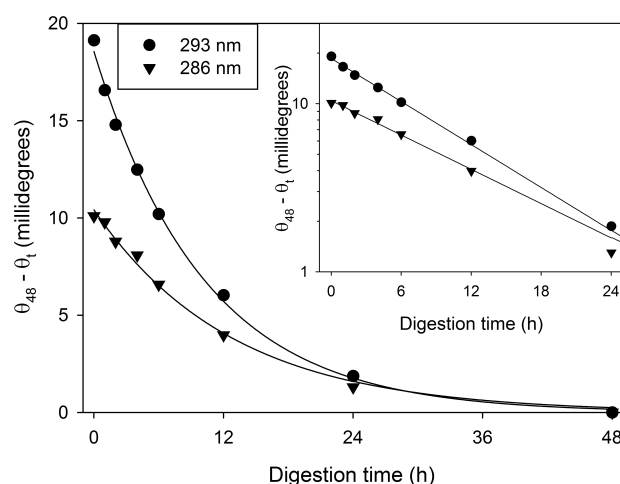

Fig. 9 Kinetics of ellipticity loss at 293 nm and 286 nm for native β-lg digested with pepsin (ratio 1:6) at 37 °C. Lines show the fit with Eq 3.

For the trough at 293 nm, values of A and B in Eq. 3 were 18.6 ± 0.3 mdeg and 0.098 ± 0.004 h^{-1} respectively. Corresponding parameters for the trough at 286 nm were 10.4 ± 0.2 mdeg and

0.078 ± 0.005 h^{-1}, a similar rate constant to that at 293 nm. Eq. 3 is entirely empirical in this context, but the excellent fit across the whole time course (Fig. 9) demonstrates consistent kinetics over 48 h, in contrast to the multiple kinetic regimes seen in Fig. 7. The near-UV region monitors the disappearance of full-length properly-folded β-lg. The half-life of this process (~8.5 h) matches closely the half-life for disappearance of β-lg, as monitored by SDS-PAGE (Fig. 6).

In contrast, there was evidence of discontinuous change in far-UV CD spectra collected during digestion (Fig. 10). Spectra changed little during the first 2h, then ellipticity at 218 nm increased substantially (i.e., became less negative) between 2 h and 4 h. This discontinuity in the far-UV CD spectra is congruent with the discontinuity observed in the SDS-PAGE analysis (Fig. 6 and Fig. 7). At longer digestion times, the trough moved to a shorter wavelength, along with the peak at ~200 nm.
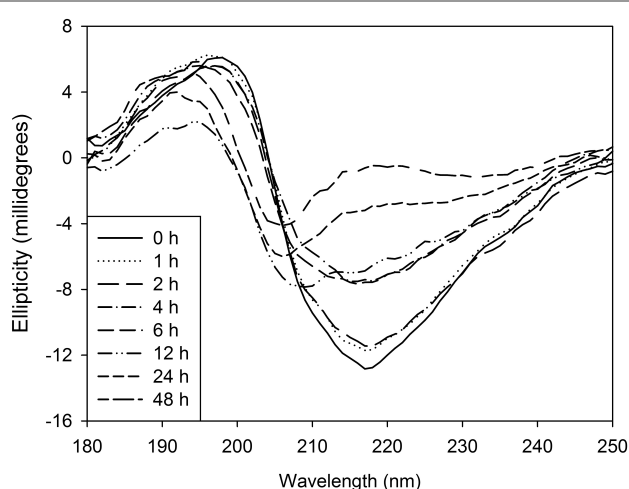

Fig. 10 Far-UV CD spectra of native β-lg during digestion with pepsin (ratio 1:6) at 37 ˚C.

These changes are consistent with an initial preservation of secondary structure (but not tertiary structure) and slow digestion of β-lg over the first several hours. In the next phase, beginning between 2 and 4 hours after addition of pepsin, helical structure is lost, indicated by the broadening of the trough and accompanied by more rapid digestion of β-lg. Secondary structure at 4 h is estimated by CAPITO[22] as 0 % helical, 49 % β-sheet and 51 % irregular; spectral similarity to the β-sheet protein immunoglobulin is observed.[23] In the final phase, beginning after 12 hours, β-lg is increasingly chewed up (at 12 h, <25 % intact and folded β-lg remaining, as indicated by Fig. 6 and Fig. 8), and hydrolysed into low molecular weight peptides that are not visible in the SDS-PAGE in Fig. 6. These peptides have substantial proportions of helical structure, e.g. at 12 h, 37% alpha-helical, 10 % beta sheet and 52 % irregular (estimated by CAPITO[22]), and visually similar to, for example, the mixed α/β pancreatic ribonuclease and β-amylase.[23] At 48 h, essentially no intact β-lg remains, as evidenced by both SDS-PAGE (Fig. 6) and near UV-CD (Fig. 8). The peptides remaining have molecular weights less than ~2 kDa and yield a

far-UV signal indicating partial helical structure with spectral similarity to, for example, ubiquitin (25 % helical, 34% β sheet and 41 % irregular[23]).

It appears that the mechanism of peptic hydrolysis does not change, since β-lg hydrolysis follows first-order kinetics in both phases, but a structural transition may facilitate a shift from slow digestion to fast digestion. This structural change is apparently not related to monomer tertiary structure. However it may stem from subtle secondary structure changes related to a shift in the monomer-dimer equilibrium that is apparent in the far-UV CD, where after a lag period of several hours ellipticity then undergoes substantial change on further digestion. This equilibrium at low pH is highly sensitive to pH and ionic strength.

The 2-phase behaviour of β-lg digestion by pepsin may be related to subtle secondary structure changes around ~4 h, but there are also another possible explanations.

First, pepsin activity rises very sharply with pH as pH increases from ~1.5, and the optimum pH for pepsin activity is 1.5-2.5[29].

We wondered if the formation of polypeptide fragments on proteolysis cause an increase of pH, leading to an increased pepsin activity. The initial pH of SGF is 1.2, but due to the buffering effects primarily of β-lg, the pH at the start of the proteolysis is 2.11, and pH rises to 2.44 over the initial 50 minutes (see ESI). Rapidly changing activity with increasing pH is therefore not the source of the two-phase behaviour.

A second possibility is that certain peptides produced by β-lg cleavage may initially bind to the active site of pepsin and inhibit its action, leading to a progressive slowing of hydrolysis in phase 1 (Fig. 7) as the concentration of uninhibited pepsin declines. This scenario is analogous to the inhibition of dipeptidyl peptidase IV by β-lg and α-lactalbumin peptides[30] and the inhibition of proteinase K by lactoferrin peptides.[31] The build-up of peptides with molecular weight ~3.4 kDa seen in SDS-PAGE (Fig. 6) is consistent with this hypothesis.

The sudden increase in β-lg hydrolysis after ~4 h (Fig. 7) may therefore result from the 'freeing up' of previously peptide-inhibited pepsin molecules, as a result of a pH-gated transition that releases the inhibitory peptide. A possible candidate for pH gating is the active-site Asp of pepsin, which under normal, i.e. non-inhibited, proteolysis has a pK_a of 1.57 ± 0.04[32]. The pK_a of this Asp may have an elevated value as a result of binding the β-lg inhibitor fragment. β-Lg itself has a pH-gated transition, the Tanford transition, for which the pK_a of the Glu responsible can be perturbed by ligand-binding in the hydrophobic cavity[33]. The peptides that built up during phase 1 subsequently disappear during phase 2 (Fig. 6), indicating that they are inherently digestible.

Conclusions

Here we have sought to integrate knowledge of heating effects on β-lg structure with an examination of how protein structure affects hydrolysis by pepsin. We have quantified heat-induced denaturation under a range of conditions, and the results support previous findings that the order of denaturation with

respect to time varies with concentration. Using a combination of SDS-PAGE and RP-HPLC we have identified peptides that resist peptic digestion and built the methodological framework for targeted structural analysis of digestion-resistant heated β-lg species.

In a surprising finding, peptic digestion of native β-lg over 48 h occurred in two distinct phases: initial digestion of up to ~15% of β-lg then a transition at ~4 h to more rapid digestion of the remaining protein. A simple first-order kinetic model fitted data in both phases. In order to explain these observations, we have a mechanism that involves pepsin inhibitory effects of β-lg peptides.

This work has unpacked some of the complexities of the processing-structure-digestibility relationship in the context of a highly simplified system. Further work is needed to characterise the structural features responsible for resistance to pepsinolysis, and to explore the implications of these findings for food processors, regulatory authorities and consumers.

Acknowledgements

Notes

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Electronic Supplementary Information (ESI) available: NUV and FUV spectra of pepsin in SGF incubated for 0-48 h; changes in pH during peptic digestion of native β-lg.

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