Nano-structuring by means of proteolysis
Rheology of novel gels from α-lactalbumin

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ABSTRACT

α-Lactalbumin (a-La), a major whey protein, has been shown to form nano-tubular gels when subjected to limited hydrolysis by a protease specific to glutamic and aspartic acid bonds. The gel stiffness of the resulting gels is extremely pH sensitive with an optimum stiffness at ~ pH 7, and the gels are also very sensitive to mechanical strain, but are able to partly regain structure under quiescent conditions.

INTRODUCTION

Within the last few years, it has become clear that the ability to form structured aggregates is an inherent property of many, if not most, proteins[1]. Many quite different proteins can be induced under appropriate conditions to form so-called amyloid fibrils and it has been suggested that the capacity for amyloid fibrillogenesis is an innate property of the peptide chain, shared by essentially all proteins[2-4]. Many proteins have a high propensity to aggregate, when in a partly folded state, and can form fibrillar or tubular structures deposited in cells impairing their normal physiological function. Improving our understanding of the mechanisms of fibril assembly can thus have significant medical implications as amyloid aggregates are characteristic of Alzheimer's disease, Parkinson's disease, the prion diseases and a number of rare neurological disorders.

We have recently discovered[5] that a-La self-assembles into long, uniform, tubular strands, about 20 nm in diameter, upon limited proteolysis by a protease from *Bacillus licheniformis* (BLP). A gel network was formed with solutions containing 3% a-La or above[6]. a-La is the second most abundant protein in the whey fraction of milk, a 123 amino acid residue compact globular protein binding one calcium molecule in a loop and stabilized by four disulfide bridges. We have applied a number of experimental techniques to study the final gels as well as the processes resulting in formation of tubular assemblies from a-La, and characterized the aggregates with respect to morphology[6,7].

However, the factors influencing the process have not been well mapped, and the present work was undertaken in order to investigate the role of pH on the gel structure building from α-La nano-tubes formed through limited proteolysis.

MATERIALS AND METHODS

α-La was prepared to a high degree of purity (*i.e.* no other proteins present as seen by capillary electrophoresis) in our laboratory using the method described previously[8]. Serine proteinase (BLP) from Bacillus licheniformis, specific for Glu-X and Asp-X bonds[9], was kindly provided by Novozymes A/S (Bagsværd, Denmark). The lyophilised enzyme preparation exhibited an activity of 14.8 CPU/g. All chemicals used

were analytical grade from Merck (Darmstadt, Germany) and highly purified water (MilliQ Plus, Millipore Corporation, Bedford, USA) was used.

Solutions of 4% α-La were made in 0.075M Tris-HCl buffer, with $CaCl_2$ added to a molar ratio of 3:1 (Ca:α-La) and stored overnight at 5°C. pH was adjusted (6.0 to 7.5) using 4M HCl. Enzyme solutions were prepared just before use by dissolving BLP in 0.5 ml of distilled water and added to 3 ml α-La solution to give a final enzyme/substrate ratio of 2%.

Dynamic oscillation (frequency 0.5 Hz, strain 0.005) was performed using a controlled strain rheometer (Bohlin VOR, Bohlin Ltd., Cirencester, UK) fitted with a C-14 measuring system. 2.8 ml of the α-La solution with added enzyme was introduced into the measuring system, which was pre-heated to 25°C. A thin layer of silicon oil was added to prevent evaporation. The sample was heated from 25 to 50°C at 1°C/min and then incubated at 50°C for 24 hrs. Following incubation, gels were cooled to 25°C at 2°C/min and a linear strain sweep was performed at 0.5 Hz using strains from 0.002 to 0.21. The build-up of the gels was subsequently followed for 30 minutes (0.5 Hz, strain of 0.005).

Three replicates were performed for all experiments.

RESULTS AND DISCUSSION

Gelation was not possible at pH values below 6.5. The time of gelation (defined as the time when the phase angle dropped below 45°) decreased as pH increased from 6.5 to 7.5 (Table 1). This is in accordance with previous results, indicating that BLP exhibits increased activity with increasing pH[10].

The stiffness of the gels resulting from treatment of α-La with BLP at different pH is illustrated in Fig. 1, and it is immediately apparent that the gels achieved maximum stiffness at a pH value of 7.0.

Table 1. Time of gelation (phase angle < 45°) at different pH values. 4% α-lactalbumin solutions incubated at 50°C with BLP. Enzyme/substrate ratio 2%.

pH	Time of gelation (min)
6.0	No gelation
6.5	86 ± 2
7	60 ± 4
7.5	58 ± 2

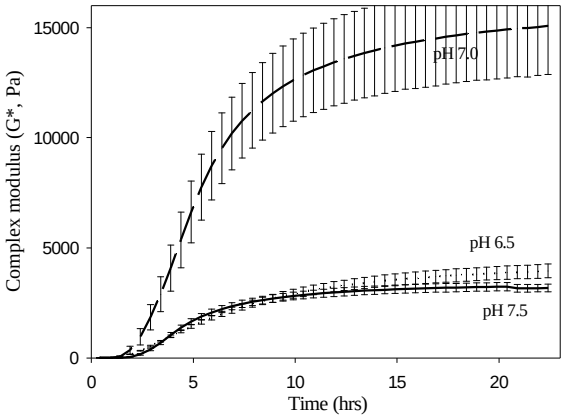

Figure 1. Evolution of the complex modulus with time during incubation at 50°C with BLP at different pH values (4% α-Lactalbumin solutions, enzyme/substrate ratio 2%).

We have proposed[6,7] that both hydrophobic and ionic interactions are important in gel formation and in assembly of the tubular structures from the peptides resulting from the proteolysis of α-La with BLP. Additionally, calcium seems crucial for the formation of gels, and presumably through mediation of ionic interactions. Ionic interactions depend greatly on pH and the maximum in gel stiffness at pH 7 possibly occurs due to optimal conditions at this pH for calcium acting to bind the peptide precursors together into tubules. The theoretical charge at pH 7 (calculated using GPMAW v. 3.04, Lighthouse Data, Odense, DK) for the dominant peptide[7] found in the tubules is –6, which could counteracted by

three Ca^{2+}, of which one is bound within the calcium-binding loop of α-La. Thus the probability exists, that at pH 7, precisely two Ca^{2+} bind to the negatively charged surface of the peptide precursor resulting from the partial hydrolysis of α-La and ensure an ordered aggregation of these peptides into stiff tubular structures. At a slightly higher pH, the peptides will be more negatively charged, resulting in increased repulsion and hence difficulty in the spatial ordering of the precursors. At pH values lower than 7, on the other hand, the peptides will have insufficient charge to bind two Ca^{2+} and hence the tubes will not be adequately bonded together.

Fig. 2 illustrates, that when subjected to a strain sweep, the gels were broken down at small strains (< 0.05). This took place irrespective of pH and is most probably due to the tubules being partially broken up into their constituent building blocks. The observed influence of pH on the mechanical breakdown of the gels can possibly be assigned to differences in the balance between hydrophobic and ionic interactions. Following strain induced breakdown, the gels exhibited partial recovery at all the investigated pH values, as indicated in Fig. 3 for a sample incubated at pH 7. Gels did not totally regain their mechanical strength within the time frame studied (30 min), but only approximately 50%. This can be assumed to be a consequence of the difficulty in building up a coherent spatial structure from a number of constituent parts that has lost their original place in the gel network. Another possible explanation could be, that the breakdown into smaller fragments results in a lower concentration of the building blocks responsible for tubule formation and hence weaker gels are obtained.

It is interesting, however, that rebuilding is possible at all, and this could possibly be utilized for technological applications in the food industry or elsewhere.

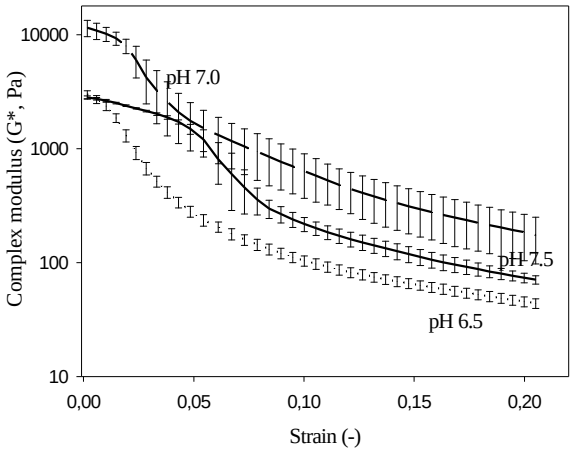

Figure 2. Strain sweep (performed after cooling to 25°C) of gels made after 24 hrs incubation at 50°C with BLP at different pH values (4% α-Lactalbumin solutions, enzyme/substrate ratio 2%).

CONCLUSION

Nano-tubular gels made from α-La through limited proteolysis are very pH sensitive and exhibit a maximum in stiffness at pH 7, presumably due to optimal conditions at this pH for the binding of Ca^{2+} to the peptide precursors hence facilitating formation of tubes.

The gels are very sensitive to strain induced breakdown, though they do, in fact, regain ~50% of their initial stiffness after 30 min under quiescent conditions.

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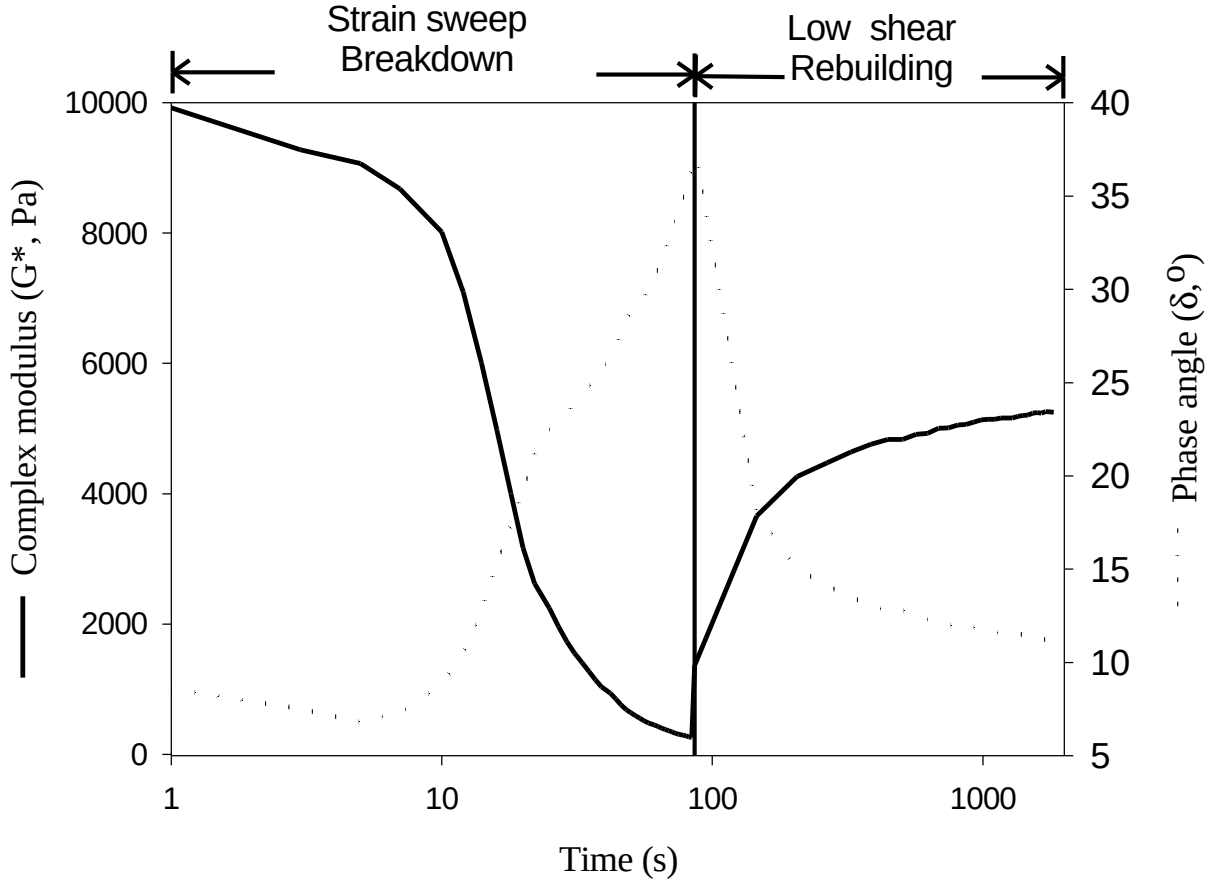

Figure 3: An example of strain induced breakdown followed by rebuilding under low strain conditions (0.005) for a gel made at pH 7, from 4% α-Lactalbumin, enzyme/substrate ratio 2%. Strain sweep was performed after cooling to 25°C on a gel made after 24 hrs incubation at 50°C with BLP.

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