Molecular self-assembly of partially hydrolysed α-lactalbumin resulting in strong gels with a novel microstructure

By RICHARD IPSEN*, JEANETTE OTTE and KARSTEN B. QVIST

Department of Dairy and Food Science, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

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Summary. Gelation of α-lactalbumin (α-la) incubated with a protease from *Bacillus licheniformis* (BLP) at 50 °C for 4 h was monitored using small oscillatory shear and the large deformation properties of final gels were characterized by uniaxial compression. Transmission electron microscopy was used to visualize the microstructure. Gels made from α-la (10 g/l) using BLP were almost transparent, although somewhat whitish, and they were more than 20 times stiffer (measured as complex modulus) than equivalent gels made from β-lactoglobulin (β-lg) at the same concentration. The microstructure of the gels consisted of non-branching, apparently hollow strands with a uniform diameter close to 20 nm, similar in overall structure to microtubules. Adding Ca^{2+} in amounts of 50 or 100 mm changed the spatial distribution of the strands and resulted in a reduction in the failure stress recorded in uniaxial compression. Apart from affecting the microstructure, Ca^{2+} was shown to be essential for the formation of the gels. It is proposed, that the mechanism behind the self-assembly of the partially hydrolysed α-la into long tubes is a spatially restricted creation of ionic bonds between Ca^{2+} and carboxyl acid groups on peptide fragments resulting from the action of BLP on α-la. Proteolysis of α-la with BLP in the presence of Ca^{2+} thus results in formation of a strong gel with a microstructure not previously observed in food protein systems.

Keywords: α-Lactalbumin, gelation, protease, fibrils, microstructure.

We have previously reported how a serine proteinase isolated from *Bacillus licheniformis* (BLP) induces aggregation and gelation in whey protein isolate (WPI) and β-lactoglobulin (β-lg) forming whitish and opaque gels (Otte *et al*. 1996*a*, 1997, 1999). In WPI that was not heat treated, the process of gelation included a rate-limiting aggregation process occurring simultaneously with the proteolysis in a manner similar to renneting of milk. A possible mechanism for the BLP-induced gelation of unheated WPI was proposed as a proteolytic release of hydrophilic as well as hydrophobic peptides from β-lg, with the latter having less electrostatic repulsion and increased possibility of hydrophobic (and other) interactions, causing them to aggregate into particles that finally constitute a gel network (Ipsen *et al*. 2000*a*).

The proteinase-induced gelation thus appeared to be dominated by the actions of the enzyme on β-lg, the major whey protein. However, initial screening of mixtures of β-lg and α-lactalbumin (α-la), the second major protein present in

* For correspondence: ri@kvl.dk

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whey, illustrated a dramatically improved gel formation when a pure preparation of α-la was used as substrate, compared with mixtures of the two proteins or a solution of purified β-lg (Ipsen *et al.* 2000*b*). It was also notable that the gels made from α-la appeared translucent, suggesting a more fine-stranded structure than in gels made from β-lg. The present investigation was undertaken in order to clarify the rheological and microstructural characteristics of gels formed by the action of BLP on a purified α-la preparation and thus possibly provide an additional basis for the use of this protein as a functional ingredient.

MATERIALS AND METHODS

Materials

α-La and β-lg were 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 by Kristiansen *et al.* (1998). The composition of the products is summarized in Table 1.

A serine proteinase from *B. licheniformis*, specific for Glu-X and Asp-X bonds (Breddam & Meldal, 1992) was kindly provided by T. Mathiassen (Novo Nordisk A/S, Bagsværd, Denmark). The lyophilized 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 in the preparation of all solutions.

Solutions of whey protein isolate and enzyme

Solutions of 100 g α-la/l were made in 0·075 M-Tris-HCl buffer, pH 7·5, with $CaCl_2$ added at levels of 0, 5, 10, 50 or 100 mM. The solutions were stored overnight at 5 °C. A solution of β-lg (100 g/l), without calcium added, was used for comparison.

The enzyme solutions were prepared just before use by dissolving 100 mg BLP powder in 0·5 ml of distilled water, and the enzyme was used at an enzyme/substrate ratio of 0·02.

Dynamic oscillation

Gelation was followed using dynamic oscillation on a controlled strain rheometer (Bohlin VOR, Bohlin Ltd, Cirencester, GL7 IYG, UK). The C-14 measuring system was used with a frequency of 0·5 Hz, and a strain of 0·005.

Solutions (3 ml) of α-la or β-lg were mixed with 33 μl enzyme solution at room temperature; 2·8 ml was then introduced into the measuring system, which was preheated 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 deg/min, held at 50 °C for 4 h and subsequently cooled to 25 °C at 1 deg/min. Three replications were performed for all experiments.

The complex modulus, G*, defined as $\sqrt{(G'^2+G''^2)}$, where G′ is the storage modulus and G″ the loss modulus, was used as an indicator of the total stiffness of the gelling samples. The time of gelation (t_g) was taken as the time when the phase angle, δ, where $\tan(\delta) = G''/G'$, dropped below 45 °.

Uniaxial compression

Protein solutions (10 ml) with 111 μl enzyme added were poured into a plastic cup (diameter 20 mm) at room temperature and incubated at 50 °C for 4 h, after which the formed gels were cooled to room temperature. Four cylindrical samples (diameter 5·4 mm, height 10 mm) were cut from each gel and subjected to lubricated uniaxial

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Table 1. *Composition of purified whey proteins prepared as described by Kristiansen* et al. *(1998) and used in the experiments described in this paper*

	α-Lactalbumin	β-Lactoglobulin
Dry matter (g/100 g)	96·8	96·8
Ash (g/100 g dry matter)	2·1	1·6
Protein (g/100 g dry matter)	98·0	97·1
Calcium (g/100 g sample)	0·73	0·03
Sodium (g/100 g sample)	0·08	0·58

compression using an Instron Universal Testing Machine (Instron Ltd, High Wycombe, HP12 3SY, UK) at room temperature and a deformation rate of 0·1 m/min. A 100 N load cell was used and the samples were compressed to 75% of the initial height. Stress (σ) was calculated taking the actual stress-bearing area into account, and Hencky strain (ϵ_H) was calculated as ln (L/L_0), where L is the height of the sample during compression and L_0 is the initial height of the sample. The fracture point was determined as the local maximum of the σ-ϵ_H curve and stress at fracture (σ_f) and Hencky strain at fracture (ϵ_{Hf}) were used as indicators of respectively the hardness and the brittleness of the gels.

Transmission electron microscopy

BLP-induced gels of α-la were made by incubating for 4 h at 50 °C followed by equilibration for approximately 30 min at room temperature. Small cubes were cut from the gelled samples and fixed in glutaraldehyde (30 g/l). Post-fixation was done using OsO_4 (10 g/l in 0·1 M-cacodylate buffer, pH 7·3). Dehydration, embedding, and cutting were performed as previously described (Otte *et al.* 1996*b*), and examination was performed using a Philips CM-100 electron microscope PW 6020 (Philips Electron Optics, Eindhoven, The Netherlands) operated at 60 kV.

RESULTS

Gel properties

α-La formed gels with BLP at all levels of Ca^{2+} added. The gelation profile of the α-la with 10 mM-Ca^{2+} is shown in Fig. 1. During the incubation period at 50 °C, the gel stiffness (G*) of all the samples continuously increased with time and δ decreased. When cooling was initiated G* initially increased, then decreased rapidly to an equilibrium value at 25 °C, whereas δ increased as soon as the cooling began, passed through a maximum when the temperature approached 25 °C, and reached an equilibrium value after holding for 30 min at 25 °C.

The gel stiffness at the end of the incubation period at 50 °C (G^*_{50}) and the maximum δ after cooling was initiated (δ_{max}), as well as the gel stiffness (G^*_{25}) and the phase angle (δ_{25}) after equilibration at 25 °C, were used together with the t_g, to characterize the gelation profiles. These characteristic parameters of the gelation curves are compiled in Table 2. It is obvious, that when gels were made without addition of calcium, then hydrolysis of α-la resulted in much stiffer gels than from β-lg. It is also noteworthy that the α-la gels were translucent, although somewhat whitish, compared with the β-lg gels, which appeared opaque and white, as previously reported (Otte *et al.* 1997).

Addition of Ca^{2+} had a strong conducive influence on the G* of the gels made from α-la (Table 2). This is in contrast to the effect of Ca^{2+} on gels from other whey proteins. Previous work (Otte *et al.* 1999) has documented the deteriorating influence of Ca^{2+} on the gel properties of enzyme-induced gels made from WPI, and in fact the

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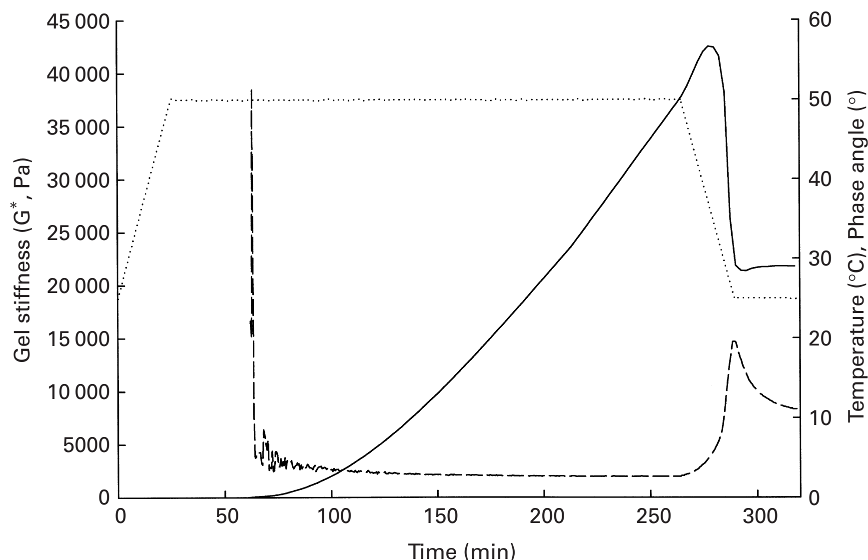

Fig. 1. An example of a gelation profile (100 g α-lactalbumin/l, 10 mM-Ca^{2+}, pH 7·5) obtained by dynamic oscillation. — Denotes the gel stiffness, ——— the phase angle and ….. the temperature.

Table 2. *Properties of gels made from α-lactalbumin with increasing concentration of Ca^{2+} or β-lactoglobulin (100 g/l) by hydrolysis using the serine proteinase BLP. G^*_{50} is the gel stiffness just after 4 h of hydrolysis at 50 °C, G^*_{25} is the gel stiffness after cooling to 25 °C and holding for 30 min, δ_{max} is the maximum phase angle after initiation of cooling, δ_{25} is the phase angle after equilibration at 25 °C for 30 min, and t_g is the time of gelation*

(Values are means of three measurements ± SD)

	G^*_{50} (kPa)	δ_{max} (deg)	G^*_{25} (kPa)	δ_{25} (deg)	t_g (min)
α-Lactalbumin					
0 mM-Ca^{2+}	9·2 ± 1·6	19·7 ± 0·5	5·3 ± 0·9	11·3 ± 0·8	78·7 ± 4·3
5 mM-Ca^{2+}	23·1 ± 0·5	18·4 ± 3·3	12·6 ± 1·0	11·1 ± 0·1	65·8 ± 0·4
10 mM-Ca^{2+}	37·6 ± 5·0	19·6 ± 2·9	21·7 ± 4·0	11·1 ± 0·1	63·4 ± 1·8
50 mM-Ca^{2+}	58·2 ± 0·6	17·5 ± 3·1	34·6 ± 0·8	11·1 ± 0·4	55·1 ± 4·7
100 mM-Ca^{2+}	49·4 ± 0·7	16·2 ± 1·5	30·3 ± 1·3	12·3 ± 0·3	71·2 ± 7·6
β-Lactoglobulin					
0 mM-Ca^{2+}	0·4 ± 0·002	n.d.	0·7 ± 0·006	4·9 ± 0·5	60·9 ± 1·1

n.d., not determined.

β-lg used in the present investigation was incapable of forming a gel when 10 mM-Ca^{2+} was added (not shown).

During cooling from the temperature of hydrolysis (50 °C) to 25 °C, a characteristic difference in the gelation profiles of α-la and β-lg could be observed: whereas G* increased for the β-lg samples, it decreased significantly for the α-la samples. Irrespective of the amount of Ca^{2+} added, the G^*_{25} was only slightly higher than 1/2 of the G^*_{50}. As the stability of hydrophobic bonds and salt-bridges in proteins decreases with decreasing temperature (Elcock, 1998), this points to a crucial role for Ca^{2+} and hydrophobic bonds in structuring the gel network formed as a consequence of the enzyme acting on α-la.

In the case of BLP-induced gels formed from β-lg, δ did not pass through a

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Table 3. *Uniaxial compression (Instron measurements) of cylinders of gels made from α-lactalbumin (100 g/l) by hydrolysis using BLP (serine proteinase from* B. licheniformis), *σ_f is stress at fracture and ϵ_{Hf} is the Hencky strain at fracture*

(Values are means of three measurements ± SD)

Added Ca^{2+} (mM)	σ_f (kPa)	ϵ_{Hf} (−)
0	4·0 ± 0·4	0·07 ± 0·0003
10	9·8 ± 1·0	0·039 ± 0·005
100	4·8 ± 0·1	0·045 ± 0·008

maximum, but decreased slightly during cooling. The stiffness of the gels made from α-la, however, still retained a final value, which was much higher than when β-lg was used. The value of δ for the gels made from α-la was close to 3° at the end of the heating period and cooling caused a marked increase (Table 2).

It should be stressed that even after 4 h of proteolysis the gel stiffness was still increasing for the samples made from α-la (Fig. 1), implying that even stronger gels can be obtained if more extensive hydrolysis is allowed to take place before cooling. Experiments, using a lower concentration of α-la, suggest that all α-la had been degraded at the time of gelation (J. Otte, unpublished results), indicating that hydrolysis of primary fragments (or assemblies of fragments) could take place after the gel point.

The G^*_{25} of the enzyme-induced gels from α-la increased with Ca^{2+} addition, at least up to 50 mM added Ca^{2+} where a remarkably high value (34·6 kPa) was obtained. The final δ was apparently not dependent on addition of Ca^{2+} (Table 2), and this indicates (van Vliet *et al*. 1989) that the bonds forming these gels are similar irrespective of the amount of Ca^{2+} added. In contrast, the δ was much lower (4·9 °) in gels made from β-lg, pointing to fundamental differences in the forces involved in formation of BLP-induced gels from these two major whey proteins.

Results from uniaxial compression measurements on enzyme-induced α-la gels are summarized in Table 3. It was not possible to make corresponding measurements on β-lg, as the gels formed were too weak to form self-supporting cylinders. The values of σ_f are within the range previously reported for 12% β-lg gels made by thermal treatment (Stading & Hermansson, 1991). Addition of 10 mM-Ca^{2+} resulted in the highest fracture stress, compared with no addition and addition of 100 mM. The measured α-la gels were all very brittle, as reflected by the low ϵ_{Hf}, and the gels made with 10 mM added Ca^{2+} were the most brittle.

It should be stressed that large deformation measurements, such as uniaxial compression, reflect the behaviour when the weakest part of a structure fails, whereas in small deformation measurements the whole structure contributes equally as no structural breakdown occurs, and these latter measurements can often be related to the properties of the individual strands making up the gel network (Stading *et al*. 1993).

Microstructure

The microstructure of the proteinase-induced gels from α-la was strikingly different from that which has been observed in other gels made from globular food proteins, which are either fine-stranded and transparent, or consist of aggregated particles forming a continuous network and opaque gels (Barbut & Foegeding, 1993; Otte *et al*. 1996*b*; Ju & Kilara, 1998). As illustrated in Fig. 2, and as expected from

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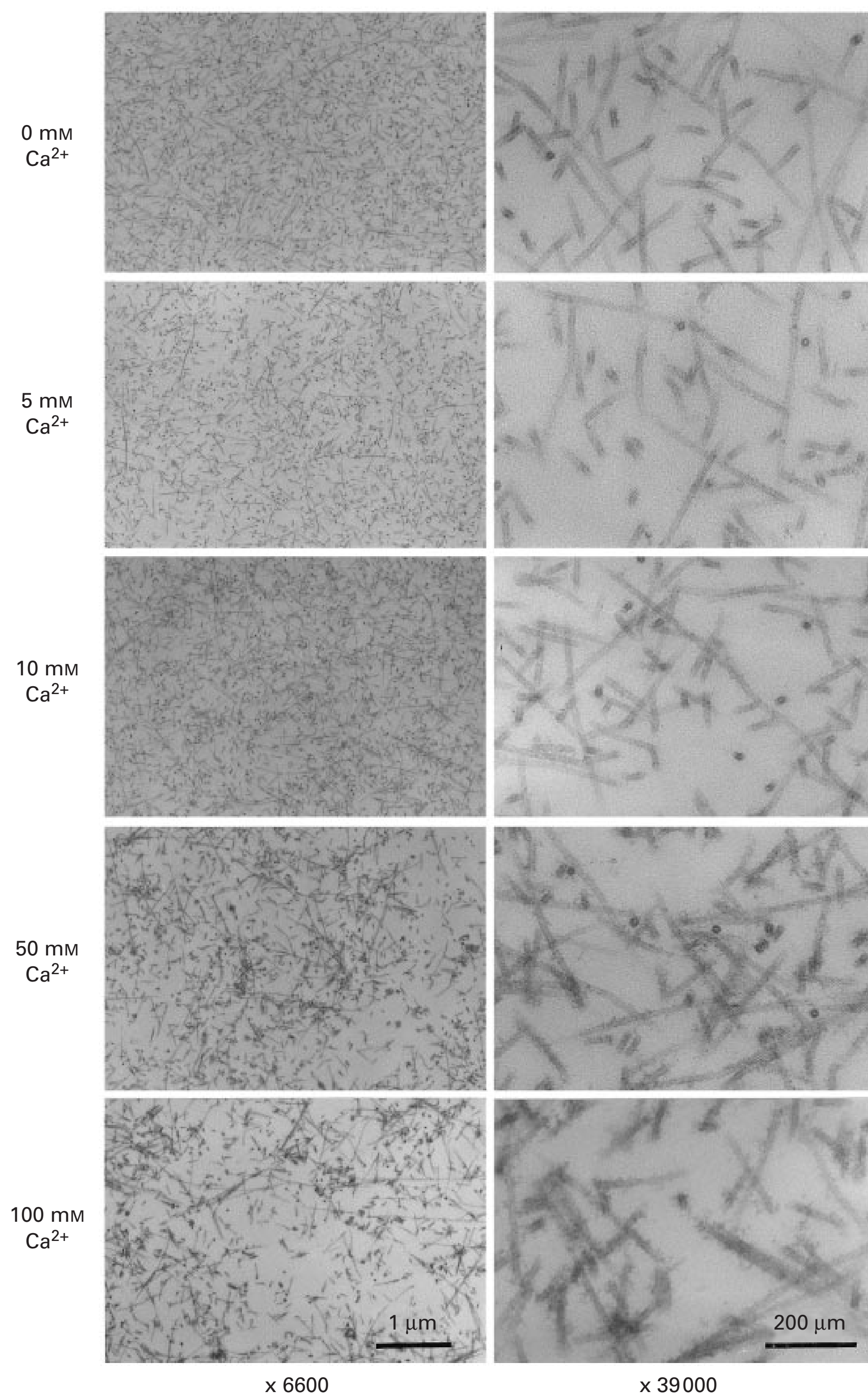

Fig. 2. Transmission electron micrographs of gels made from 100 g α-lactalbumin/l, pH 7·5, with various levels of added Ca^{2+}. Gels are shown at low (6600 ×; left) and high (39000 ×; right) magnification.

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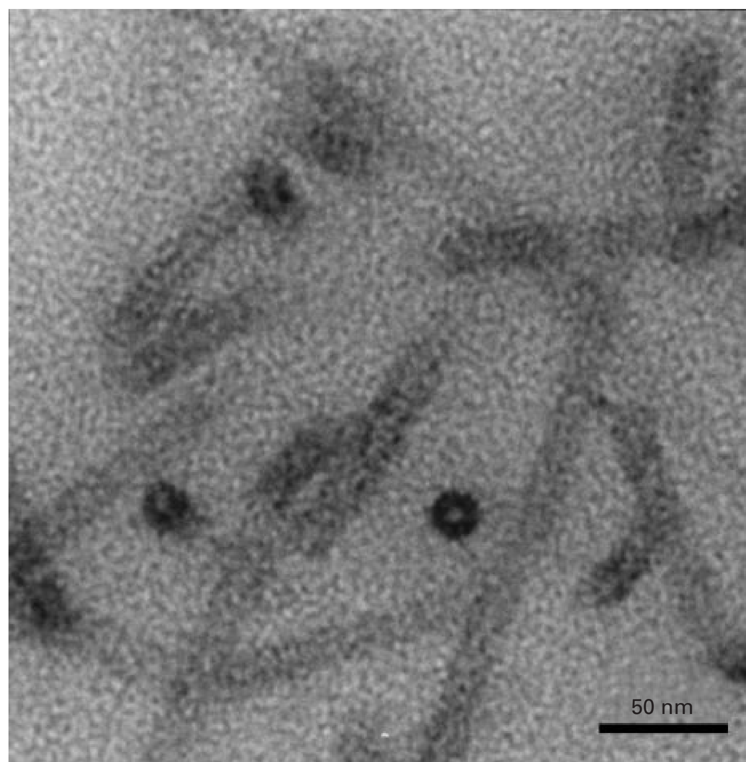

Fig. 3. Transmission electron micrograph of a gel made from 100 g α-lactalbimin/l, pH 7·5, with addition of 10 mM Ca^{2+}. Gel shown at very high (145 000 ×) magnification.

their translucent nature, the network structure of the gels formed in the present study was dominated by fine strands, some having lengths exceeding 2 μm. These strands exhibited a surprising uniformity in that all the strands were practically straight with a diameter close to 20 nm throughout their length, and no branching of the strands was apparent, although overlapping of strands was frequent. Strands cut transversally appear as small circles at high magnification (Fig. 2, right), suggesting that the strands were hollow, which was consistent with the fact that longitudinally cut strands appeared as two electron-dense lines separated by less electron-dense material. Thus limited hydrolysis of α-la with BLP resulted in formation of long tubes, and at very high magnification (Fig. 3) a transverse cut through an individual tube illustrates that the tube wall seemed to be made up of a number of individual subunits.

Addition of 5 or 10 mM-calcium did not visibly affect the microstructure of the gels, whereas amounts of 50 or 100 mM resulted in a marked change in the microstructure. At lower magnification (Fig. 2, left) it can be seen that calcium caused the individual strands making up the gel-network to associate and form regions with a denser network, accompanied by concomitant formation of regions with a smaller density of network strands, i.e. pores. When viewed at a higher magnification (Fig. 2, right), it is obvious that addition of calcium caused the individual strands to become fuzzier, possibly due to association of other released peptides to the network through formation of additional Ca^{2+} bridges.

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DISCUSSION

Molecular self-assembly of fragments stemming from partial proteolysis of a milk protein into long, unbranched tubes in turn making up a gel-network has not to our knowledge been reported previously. Molecular self-assembly is, however, ubiquitous in nature, and in the last few years considerable advances have been made in the use of synthetic peptides as building blocks to produce biological materials with potential in a number of applications, including platforms or scaffolding for tissue engineering, drug delivery of protein and peptide medicine, as well as biological surface engineering (Aggelli *et al.* 1997; Zhang & Altman, 1999). In addition, artificial proteins can be produced that form fibrils and they are believed to assemble by making a rapid transition from an α-helix to a β-sheet conformation brought about by changes in temperature or pH, reinforcing the hypothesis that when proteins lose their folded native structure, they can self-assemble into insoluble fibrils (Aggelli *et al.* 1999). Lysozymes, which are homologous to α-la (Anderson *et al.* 1997), have also been shown to be able to form highly organized amyloid fibrils (Krebs *et al.* 2000; Morozova-Roche *et al.* 2000) under conditions where partial unfolding of the native globular fold takes place (heating of low-pH solutions, addition of organic solvents).

The structure reported here, however, is tubular and exhibits similarities to the tubular protein structures (microtubules) that form an integral part of the cytoskeleton and are composed of hetero dimers of tubulin (Vértessy *et al.* 1996). Tubulin is known to self-assemble in vitro, although, in stark contrast to our system, Ca^{2+} in small amounts (6 μM) inhibits tubuli assembly (Xu, 1998). Ca^{2+} has, however, been shown to induce filamentous structures (Löwe & Amos, 1999) in a bacterial protein (FtsZ) related in structure and function to tubulin.

From the microstructure of the α-la gels made in the present study, it seems reasonable to expect that the individual, tubular strands have a high stiffness as a consequence of the intermolecular bonds involved in strand formation. This is confirmed from the measurements made at small deformations, where the total stiffness was found to be more than 20 times larger than that of equivalent β-lg gels, which are known to be built from aggregates (Otte *et al.* 1996*b*). A network composed of very stiff and inflexible individual strands would also be expected to be brittle, and this was indeed the case as evidenced by the small fracture strains recorded in uniaxial compression (Table 3).

The marked decrease in gel stiffness upon cooling observed for the α-la gels (Table 2) points to the importance of electrostatic interactions in maintaining network structure, presumably through salt-bridges with Ca^{2+}. Decreasing temperature is known to increase the electrostatic desolvation penalty incurred in forming a salt-bridge, leading to a concurrent decrease in salt bridge stability (Elcock, 1998). This is in strong contrast to what has been observed for β-lg, where hydrophobic interactions are believed to be of paramount importance (Otte *et al.* 1997; Ipsen *et al.* 2000*a*). The temperature dependence of the phase angle, most notably the observed maximum during cooling from 50 to 25 °C, corroborates this and highlights the different nature of BLP-induced gels made from α-la and β-lg.

Our results are unambiguous as to the great significance of calcium in formation of the gel network that follows proteolysis with BLP. Negatively charged carboxylic acid groups are formed by the action of this enzyme, as it specifically cleaves peptide bonds containing an acidic amino acid (Glu or Asp, with a preference for the former). We propose that ionic bonds, involving Ca^{2+} and these negatively charged groups,

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are the primary factor responsible for the formation of the observed uniform strands that ultimately result in gels with the reported novel microstructure. The 10% α-la solutions used had a content of Ca^{2+} equivalent to 17·6 mM and the molar ratio of Ca^{2+} to α-la was 2·6. A similar preparation with a molar ratio of only 1·1 was not able to form a gel upon hydrolysis with BLP, but when Ca^{2+} was added to a molar ratio of 2·6, gelation was induced (R. Ipsen, unpublished results), proving that Ca^{2+} is, in fact, necessary for gel formation to take place, and pointing to about two molecules of Ca^{2+} to every molecule of α-la being prerequisite for gel formation.

Adding Ca^{2+} in excess of the amount required for gel formation lowered the t_g and increased the stiffness of the individual strands (Table 2). As no branching of the tubular strands took place, and there was no observable difference between the microstructure of gels at levels of 0–10 mM added Ca^{2+}, nor any significant differences between the phase angles of the final gels, we believe that this increase, taking the difference in the equilibrium modulus into consideration, was due to formation of additional electrostatic bonds between Ca^{2+} and the peptide units within the strands. The maximum gel stiffness obtained at 50 mM added Ca^{2+}, where denser regions of associated strands were observed (Fig. 2), was presumably caused by Ca^{2+} forming bridges between different strands. The observed fuzziness of the gel network at this Ca^{2+} concentration (and at 100 mM added Ca^{2+}) (Fig. 2) could be due to association of additional peptides released upon hydrolysis to the network. An adverse consequence of adding Ca^{2+} in amounts of 50–100 mM compared with an addition of 10 mM-Ca^{2+}, was the appearance of pores in the gel, caused by association of individual strands, and this was reflected in a decreased failure strength (σ_f) (Table 3).

The striking uniformity of the tubes acting as structural elements in the obtained gels implies that the units initially building the tubes must be subjugated to strong spatial restrictions. Partial hydrolysis of α-la is a prerequisite for gelation, as Ca^{2+} in itself does not induce gelation. Probably, enzymic cleavage results in release of a few peptide fragments from the original α-la molecule, allowing the remaining molecule to associate through formation of Ca^{2+} bridges. α-La is known to contain a primary Ca^{2+} binding site (Aramini *et al*. 1996) as well as an additional low-affinity site (Chandra *et al*. 1998; Hendrix *et al*. 2000). It is tempting to suggest that hydrolysis with BLP results in exposure of the calcium-binding loop in α-la, hence providing the possibility of sharing Ca^{2+} with other molecules. However, we do not know at present whether the individual strands in the gel network essentially consist of the same fragment of α-la. It is quite possible that the Ca^{2+}-stabilized fragments of α-la, produced as a result of the hydrolysis, are all relatively similar in size and composition. This contrasts with the mix of peptides obtained from β-lg (Otte *et al*. 1997), and could explain the differences in the gelation behaviour of these proteins. We expect, though, that the self-assembly of the structural elements from α-la is a highly ordered process, perhaps with similarities to what has been observed in artificial protein and peptide systems and in naturally occurring tubular protein structures. We are at present investigating the outcome of hydrolysis of α-la by BLP in order to identify the fragments involved in formation of tubes, and hence move further towards an understanding of the mechanism behind this intriguing process.

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