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β-Lactoglobulin nanofibrils: Effect of temperature on fibril formation kinetics, fibril morphology and the rheological properties of fibril dispersions

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ABSTRACT

Almost all published studies of heat-induced β-lactoglobulin self-assembly into amyloid-like fibrils at low pH and low ionic strength have involved heating at 80 °C, and the effect of heating temperature on self-assembly has received little attention. Here we heated β-lactoglobulin at pH 2 and 75 °C, 80 °C, 90 °C, 100 °C, 110 °C or 120 °C and investigated the kinetics of self-assembly (using Thioflavin T fluorescence), the morphology of fibrils, and the rheological properties of fibril dispersions.

Self-assembly occurred at all temperatures tested. Thioflavin T fluorescence increased sigmoidally at all temperatures, however it decreased sharply with >3.3 h heating at 110 °C and with >5 h heating at 120 °C. The sharp decreases were attributed partly to local gelation, but destruction of fibrils may have occurred at 120 °C. Thioflavin T fluorescence results indicated that maximal rates of fibril formation increased with increasing temperature, especially above 100 °C, but fibril yield (maximum Thioflavin T fluorescence) was not affected by temperature.

At 100 °C and 110 °C, fibrils were slightly shorter than at 80 °C, but otherwise they looked very similar. Fibrils made by heating at 120 °C for 1 h were also similar, but heating at 120 °C for 8 h gave predominantly short fibrils, apparently the products of larger fibrils fragmenting. Heating at 100 °C gave consistently higher viscosity than at 80 °C, and heating for >2 h at 120 °C decreased viscosity, which may have been linked with fibril fragmentation seen in micrographs.

1. Introduction

Amyloid fibrils consisting of stacked protein β-sheet structures are seen *in vivo* in association with certain degenerative diseases called amyloidoses. Structurally similar 'amyloid-like' fibrils form *in vitro* under certain denaturing conditions, such as high temperature with low pH and low ionic strength, or in high concentrations of alcohols (Gosal, Clark, & Ross-Murphy, 2004). A wide range of proteins form amyloid fibrils, including a number that are not associated with amyloidoses, which has lead to the suggestion that amyloid formation is a generic property of polypeptide chains (Dobson, 1999). Amyloid-like fibrils may be useful as scaffolds for enzyme immobilisation (Pilkington, Roberts, Meade, & Gerrard, 2009) or tissue engineering (Yan et al., 2008). The food industry is also interested in amyloid-like protein fibrils, which enhance viscosity and form gels at relatively low protein concentration (Loveday, Rao, Creamer, & Singh, 2009).

β-Lactoglobulin comprises approximately 50% of whey proteins in bovine milk, and forms amyloid-like fibrils within a few hours on heating (typically at 80 °C) at low pH and low ionic strength. Heat-induced fibril formation by β-lactoglobulin usually shows an initial lag phase when followed with the Thioflavin T (ThT) fluorescence assay (Loveday et al., 2010), in contrast to egg proteins and bovine serum albumin (Pearce, Mackintosh, & Gerrard, 2007). The limiting process during the lag phase is thought to be the formation of fibril nuclei, since the lag phase is reduced or abolished by shearing and/or adding fibril seeds (Bolder, Sagis, Venema, & van der Linden, 2007a; Hill, Krebs, Goodall, Howlett, & Dunstan, 2006). However the details of the nucleation process remain elusive. Although fibrils formed after heating for 20 h at 80–85 °C are comprised of peptides (Akkermans et al., 2008; Oboroceanu, Wang, Brodkorb, Magner, & Auty, 2010), it has not been shown whether hydrolysis is a necessary precursor to the nucleation or growth of fibrils.

It is clear that there is a common requirement among amyloid-forming globular proteins for some degree of unfolding before self-assembly into amyloid fibrils will occur (Chiti & Dobson, 2006). However, Chiti and Dobson (2006) suggested that, rather than a single assembly pathway leading to amyloid formation, "each

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protein sequence can form a spectrum of structurally distinct fibrillar aggregates and that kinetic factors can dictate which of these alternatives is dominant under given circumstances." The circumstances in our case are low pH, low ionic strength and high temperature, and the kinetic factor of interest in the present work is the effect of heating temperature.

Changing the temperature of an aqueous solution of a globular protein changes the balance of forces determining the position of the equilibrium between folded and unfolded states. In the folded state, there are internal hydrogen bonds, van der Waals interactions and often disulphide bonds stabilising the structure, and most hydrophobic regions are buried in the interior, minimising the entropically unfavourable interaction (at ambient temperature) between hydrophobic residues and water molecules (Privalov & Makhatadze, 1993). The transition to a completely unfolded state involves breakage of internal bonds, unfolding of the peptide chain, and hydration of previously buried residues, all of which contribute in a temperature-dependent way to the Gibbs free energy of unfolding (Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993).

The net result is that the Gibbs free energy of unfolding for globular proteins has a parabolic dependence on temperature (Franks, 2002; Privalov & Makhatadze, 1993), and becomes negative (spontaneous unfolding) above the thermal denaturation temperature and below the cold denaturation temperature. Above the thermal denaturation temperature, the Gibbs free energy of unfolding becomes more negative with increasing temperature (Privalov & Makhatadze, 1993), and this translates to an increased rate of denaturation. For β-lactoglobulin at pH 2.5 and low ionic strength, the pseudo-first order rate of unfolding was minimal below 75 °C and increased dramatically between 85 °C and 94 °C (Harwalkar, 1980).

Once unfolded, a variety of protein–protein crosslinks can form (Gerrard, 2002). During heating at low pH, disulphide bonding between β-lactoglobulin molecules does not occur to any significant extent (Alting, de Jongh, Visschers, & Simons, 2002; Otte, Zakora, & Qvist, 2000) because cysteine residues are predominantly protonated. Even with 20 h heating at 85 °C and pH 2, Akkermans et al. (2008) reported several suspected chemical modifications to amino acid side chains, but no covalent crosslinking.

Besides crosslinking, another chemical reaction that can occur at low pH and high temperature is hydrolysis of peptide bonds. Acid-catalysed hydrolysis of amides, such as the peptide bond, proceeds *via* protonation of the carbonyl oxygen followed by rate-limiting nucleophilic attack on the amide carbon by a water molecule oxygen (Brown, Bennet, & Slebocka-Tilk, 1992). Steric effects of amino acid side chains on either side of a peptide bond affect the susceptibility of the amide carbon to attack by nucleophiles, so the rate of cleavage of a given peptide bond depends on both the identity of the amino acids involved and their order (Harris, Cole, & Pon, 1956). Kinetic studies with dipeptides indicated that peptide bonds involving C-terminal aspartic acid (Asp) residues were particularly labile in hot acid (Harris et al., 1956). This was confirmed by the appearance of peptides with N-terminal Asp in protein acid hydrolysis reactions (Akkermans et al., 2008; Frare, de Laureto, Zurdo, Dobson, & Fontana, 2004; Inglis, 1983; Schultz, 1967). Peptides with C-terminal Asp residues were also common products in these studies, indicating that both Asp-X and X-Asp peptide bonds (where 'X' is any other amino acid) are easily cleaved in hot acid. Increasing the temperature of an acidic peptide solution increases the rate of peptide bond cleavage, but apparently does not change the relative susceptibilities of peptide bonds between different amino acid pairs (Harris et al., 1956; Lawrence & Moore, 1951).

Amyloid-like fibril formation by β-lactoglobulin is preceded by the formation of oligomeric 'seeds', typically 28 nm wide and 4–5 nm high (Hill et al., 2006). Addition of an extra building block onto a nucleus or fibril requires the two to diffuse together in solution, so the viscosity of the solution will have a non-specific effect on fibril nucleation and growth. The viscosity of water decreases from 374 μPa s at 75 °C to 230 μPa s at 120 °C (Cooper & LeFevre, 1969), so diffusion of monomers and growing species is expected to be faster at higher temperatures. Increased thermal motion at higher temperature may also speed up alignment/rearrangement and attachment of the extra building block, but this effect is hard to quantify.

Previous reports of heat-induced fibril formation experiments with β-lactoglobulin have been carried out almost exclusively at pH 2 and 80 °C. We have not found systematic studies of how heating temperature affects the heat-induced self-assembly of β-lactoglobulin at low pH and low ionic strength. In an earlier paper (Loveday et al., 2010) we explored the effect of pH, NaCl and $CaCl_2$ concentration on fibril formation kinetics and fibril morphology. Here we examine the effect of temperature on the kinetics of fibril formation and morphology of fibrils. We also report the effect of heating time and temperature on the viscosity of β-lactoglobulin nanofibril dispersions.

2. Materials and methods

2.1. Chemicals

Thioflavin T powder and β-Lactoglobulin (90% pure), containing a mixture of genetic variants A and B, were purchased from Sigma–Aldrich (St. Louis, MO). Milli-Q® water was used throughout the study.

2.2. Preparation of β-lactoglobulin solutions

Milli-Q water was adjusted to pH 2 ± 0.05, and β-lactoglobulin was added to make a 1.2% w/v dispersion. The solution was stirred overnight at 4 °C then centrifuged at 22,600 × g for 30 min (Himac CR22G II super speed centrifuge, Hitachi Koki Co., Japan) and filtered (0.2 μm syringe filter, Millex-GS®, Millipore, Billerica, MA).

Residual salts were removed by ultrafiltration using a centrifugal filter (10 kDa cutoff, Amicon® Ultra-15, Millipore) by centrifuging at 3000 × g for 15 min. Filters were rinsed with pH 2 water prior to use. The retentate was topped up to the original volume with pH 2 water, and filtering was repeated two more times. After three filtration steps, the conductivity of the protein solution was close to that of the pH 2 water.

Protein concentration in the desalted β-lactoglobulin solution was determined by absorption at 278 nm (Ultrospec 2000 UV spectrophotometer, Pharmacia Biotech, Cambridge, UK), using a β-lactoglobulin standard curve and assuming 90% purity, as stated by the supplier. A small proportion of protein was lost during centrifuging and filtering, and an initial concentration of 1.2% w/v gave a final concentration close to 1% w/v. Solutions of β-lactoglobulin were stored at 4 °C and used within two days of preparation.

2.3. Heating of β-lactoglobulin solutions

Aliquots of β-lactoglobulin solution in screw-capped glass tubes (16 mm diameter Kimax® glass, Schott, Elmsford, NY) were heated in a dry block heater (Techne DB-3D, Bibby Scientific Ltd, Staffordshire, UK). Following the requisite heating time, a tube was cooled in ice water for 5–10 min. Aliquots were 2 mL for the ThT assay and 6 mL for rheometry.