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Review

Kinetics of spherulite formation and growth: Salt and protein concentration dependence on proteins β-lactoglobulin and insulin

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ABSTRACT

Proteins aggregated into spherulite structures of amyloid fibrils have been observed in patients with certain brain diseases such as Alzheimer's and Parkinson's. The conditions under which these protein spherulites form and grow are not currently known. In order to illuminate the role of environmental factors on protein spherulites, this research aims to explore the kinetics and mechanisms of spherulite formation and growth, as monitored by optical microscopy, in a range of salt concentrations, and initial protein concentrations for two model proteins: bovine β-lactoglobulin and insulin.

These two proteins are significantly different in their size and fibril growth rate, but both of these proteins have been shown previously to form amyloid fibrils and spherulites under low pH conditions. The growth pattern of spherulites in each protein solution was monitored and quantified using a linear polymerisation reaction model which allowed for quantification of formation and growth rates across experiments.

Two themes were found in the experimental results of spherulite formation and growth: the two model protein systems behaved very similarly to one another when viewed on relative scales, and the spherulites in these systems followed trends seen in some of the previous research of amyloid fibril growth.

Specifically, in the presence of salt, both β-lactoglobulin and insulin systems demonstrated maximum growth rates at the same salt concentration, possibly suggesting the role that salt plays in altering rates may not be protein specific (e.g. anion binding to aid unfolding), but may be generic (e.g. electrostatic shielding of repelling charges).

Specifically, with variations in the initial protein concentrations, spherulite trends across both model systems were a decrease in appearance time (faster appearance) and an increased growth rate as concentration increased. The appearance time decreased at a diminishing rate towards a limiting shortest appearance time. A limiting shortest appearance time suggests that, in the higher concentrations of protein tested, spherulite formation is not dependent upon the spatial concentration of protein but on the preparedness of the protein to form or join the spherulite.

Contents

1. Introduction 302
2. Method 302
3. Results and discussion 302
   3.1. Salt concentration effects on spherulite growth 304
      3.1.1. Comparison of effect of salt concentration on β-lactoglobulin and insulin protein systems 306
   3.2. Protein concentration effects on spherulite growth 307
      3.2.1. Comparison of effect of protein concentration on β-lactoglobulin and insulin protein systems 309
4. Conclusions 309
   Acknowledgements 310
   References 310

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1. Introduction

Amyloid fibril protein aggregation and deposition has been found in several types of neurodegenerative diseases [1–10]. Furthermore, it has been observed in some instances that the protein amyloid fibrils form more complex agglomerates, described as gels, plaques, or spherulites [1,3,9,11–17]. One such complex agglomerate, spherulites, is the focus of this research. Spherulites have been found in some *in vivo* tumours and occasionally present in the brains of patients with diseases such as Alzheimer's and Downs' syndrome [6,11,14,18–21]. In addition to the observation of spherulites in *in vivo* aggregates, it has been observed that some proteins not associated with disease can form spherulites *in vitro* [22–25]. Two of these protein systems, β-lactoglobulin and insulin, are used as *in vitro* models in this research. Modifications in salt concentration and protein concentration for *in vitro* experiments, the primary focus of this research, have been observed to affect protein aggregation mechanisms [26–34]. The addition of salt is known to alter the rate of amyloid fibril formation and growth in a number of protein systems and the variation induced by the presence of salt is commonly attributed to electrostatic shielding and/or anion binding [28–31,34,27].

Specifically, previous research has shown that β-lactoglobulin fibril formation in the presence of NaCl qualitatively increased fibril branching and fibril flexibility [28]. This variation in fibril behaviour in the presence of salt was attributed to the salt screening repulsive electrostatic forces that kept the protein assembly aligned straight.

The behaviour of insulin protein assemblies in the presence of salt has been previously researched in more depth than β-lactoglobulin. Insulin amyloid fibril formation and growth at low pH (1.6) conditions has been monitored by ThT fluorescence in the presence of a range of concentrations of three different salts [30]. Nielsen et al. [30] monitored the ThT binding of insulin in the presence of either NaCl, Na_2SO_4, or NaH_2PO_4 each in concentrations of 0.05 M, 0.10 M, 0.20 M, and 0.50 M. In the presence of NaCl, the fibril formation time and rate of growth after formation both demonstrated significant decrease as the concentration of salt increased. The trends of fibril formation time and rate of growth demonstrated inconsistent trends with changing salt concentrations for the other two salts with the exception of a decreasing fibril formation time with increasing concentration of NaH_2PO_4 [30].

Insulin spherulite formation and growth has also been investigated at low pH (2.0) conditions by ThT fluorescence in the presence of NaCl in the range of 0–200 mM [29]. Krebs et al. [29] found that increasing the salt concentration within this range decreased the fibril formation time (consistent with Nielsen et al. [30]) and increased the rate of growth of the spherulites (inconsistent with the fibril results of Nielsen et al. [30]). The final images of insulin spherulites exhibited an amorphous "core" of material surrounded by a halo of radially oriented fibrils (cores were not seen in β-lactoglobulin). The resulting insulin spherulites in the presence of salt had smaller cores in proportion to the total spherulite size and had smaller overall spherulite size as well when compared to insulin spherulites formed in the absence of salt.

The influence of protein concentration in solution on the structure and kinetics of aggregation into amyloid fibrils has been investigated previously for β-lactoglobulin [26], insulin [30,32], and a related protein: β_2-microglobulin [27]. In the case where Arnaudov et al. investigated β-lactoglobulin [26,27], three β-lactoglobulin concentrations were investigated with each technique in the range of 1.0–8.2 wt%. Quantitatively, the rate of aggregation increased as the initial concentration of protein increased. Qualitatively, no significant difference in resulting fibril structure was noted by the atomic force microscopy analysis performed [27].

The influence of protein concentration on the rate of insulin aggregation has been investigated for individual fibrils [30] and for preformed spherulites [33]. In the research by Neilsen et al. [30], the quantity of insulin in solution was varied between 0.02 wt% and 2.0 wt%. As the concentration of insulin increased, the nucleation time decreased with a logarithmic dependence and the apparent rate constant of fibril growth increased linearly. These results were described as being consistent with a first order nucleation-dependent elongation reaction model [30]. In the research by Rogers et al. [33], preformed insulin spherulites were added to fresh solutions of various concentrations of insulin and then the initial rate of continuing growth of the preformed spherulites was monitored with optical microscopy. The fresh insulin solutions ranged from 0.02 wt% to 0.50 wt%. For fresh insulin concentrations of less than 0.10%, the initial apparent rate of continuing spherulite growth increased linearly with increasing concentration (consistent with Neilsen et al. [30] for individual fibrils). For concentrations between 0.10% and 0.20%, no increase in apparent spherulite growth rate was found (inconsistent with Neilsen et al. [30] for individual fibrils). This two regime phenomena of continued spherulite growth was suggested to be due to a diffusion-limited regime at low concentration and a reaction rate-limited regime at concentrations greater than 0.10% [33].

In this research, the appearance and growth of spherulites in both β-lactoglobulin and insulin low pH (1.6) protein solutions are monitored directly with timelapse optical microscopy and quantified for a range of salt concentrations and protein concentrations. Utilizing a linear polymerisation model of spherulite growth, growth rate constants were determined and compared across the environmental conditions and across the two model proteins. In this way, understanding of spherulite formation mechanisms and growth was furthered.

2. Method

The two proteins used were obtained from Sigma–Aldrich (Gillingham, UK) and were of analytical grade: β-lactoglobulin (product number L0130; mixture of types A and B), and bovine insulin (product number I5500). Protein solutions were made by dissolving the protein in distilled and de-ionised water to make β-lactoglobulin solutions of between 1 wt% and 4 wt% and to make insulin solutions of between 0.1 wt% and 3 wt%. These ranges of protein concentrations were used because at lower concentrations, spherulites were not regularly viewed in the solutions and at higher concentrations the solution turned to a viscous gel that was not conducive to viewing with optical microscopy. In the salt concentration experiments, NaCl salt was dissolved into the protein solution in concentrations of 0 M, 0.0025 M, 0.005 M, 0.01 M, and 0.025 M. This concentration was selected as a maximum because above this threshold the solution became cloudy and difficult to view with optical microscopy. The desired pH (1.6) was achieved by adding 1 M HCl (Sigma–Aldrich) in 5–50 μl increments. Glass slides with wells were filled with 100 μl of protein solution, covered with a glass coverslip, and heated at 75 °C for insulin and 80 °C for β-lactoglobulin on a heating stage placed within the Zeiss Axioplan optical microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). Total magnification of either 50× or 100× was used. A polariser and analyzer were put in fixed positions, orthogonal to one another for the cross polariser imaging. Timelapse and still images were taken using a Kodak digital camera mounted on the top of the microscope. The experimental set-up for heating and imaging the protein solutions is the same as that published [35].

3. Results and discussion

The β-lactoglobulin and insulin spherulites were observed to have a "lag time, growth, plateau" growth pattern which has