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Formation of Amyloid-like Fibrils by Ovalbumin and Related Proteins under Conditions Relevant to Food Processing

F. GRANT PEARCE,*,† SARAH H. MACKINTOSH,‡ AND JULIET A. GERRARD†

School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch 8020, New Zealand, and New Zealand Institute for Crop & Food Research Ltd, Private Bag 4704, Christchurch, New Zealand

Protein aggregation is important in food processing, and this work investigated the aggregation of food proteins as a source of amyloid fibrils for use in bionanotechnology. Both purified and crude mixtures of albumin proteins were denatured by heat, which caused aggregation to occur. Protein denaturation was measured by using circular dichroism spectrometry and by following thioflavin T fluorescence, which is widely used as a diagnostic test for amyloid formation. There was a good correlation between the increase in thioflavin T fluorescence and loss of helical structure as the temperature was increased. Formation of thioflavin T fluorescence was dependent on temperature, but less dependent on salt and protein concentration. X-ray fiber diffraction patterns of denatured bovine serum albumin suggested that the protein had a similar cross-β structure to that of amyloid fibrils. These results are consistent with the aggregates seen during food processing, being amyloid-like in nature.

KEYWORDS: Ovalbumin; bovine serum albumin; amyloid fibril formation; heat denaturation

INTRODUCTION

Protein aggregation in food has been studied for many years, with controlled aggregation being an essential part of many food processing regimes (*1*). In the wider biochemical and biomedical literature, protein aggregation is becoming an increasingly important topic, with the implication of a particular form of aggregated protein—the amyloid fibril—and its precursors in the pathology of an increasing number of human diseases (*2*, *3*). Although interest originally arose in the context of disease states, amyloid or amyloid-like fibrils have been found to play a useful biological role in spiders webs (*4*), moth silk (*5*), curli protein in bacteria (*6*), Sup35 protein in yeast (*7*, *8*), bacterial inclusion bodies (*9*), and recently in melanosomes of humans (*10*). Drawing on nature's examples, protein fibrils are also being explored as versatile components for bionanotechnology (*11*). To our knowledge, aggregated food protein has not previously been investigated directly as a source of amyloid fibrils. In this paper, we examine model food proteins for their propensity to form amyloid-like structures under conditions commonly associated with food proteins (*11*).

Albumin proteins were selected as our model since they undergo heat coagulation, and the literature on their aggregation is quite comprehensive. Ovalbumin is the major protein present in egg white, while serum albumin is found in red meat, which is often the main source of dietary protein. Previous studies have observed the formation of amyloid type cross-β structure by ovalbumin, as judged by fluorescence with thioflavin T (*12*), but the exact nature of the structural changes is unknown; in particular, the definitive test for amyloid fibril formation—observation of the cross-β structure by X-ray fiber diffraction (*13*)—has not been observed. When heated at neutral pH, ovalbumin forms soluble aggregates that have a regular structure (*14*) which Raman spectroscopy revealed to have an intermolecular cross-β structure (*15*). Circular dichroism (CD) spectroscopy also showed the formation of a β-sheet structure during heat denaturation, which increases with salt and protein concentration (*16*), and changes in the CD spectra were associated with an increase in thioflavin T fluorescence (*12*).

Electron microscopy has shown that both ovalbumin and bovine serum albumin form a gel upon heating, which is composed of a network of linear aggregates (*17*). Bovine serum albumin has also been shown to irreversibly form an intermolecular β-sheet on heating above 70 °C (*18*), despite the protein having no β-sheets in its native form.

To establish whether the aggregates previously noted in the literature were amyloid-like in nature, we have examined the denaturation that occurs when ovalbumin or bovine serum albumin is heated. Additionally, we compared the differences between purified ovalbumin and crude hen egg white, to establish whether those aggregates observed in a laboratory context are able to form in the crude protein mixtures used in a food processing context. Ovalbumin is the main constituent of hen egg white, but comprises only 54% of the total protein (*19*). Most amyloid fibrils reported in the literature, other than those that have formed in tissues during aging, have been generated from purified mixtures (*20*, *21*). There are many

* To whom correspondence should be addressed. E-mail: grant.pearce@canterbury.ac.nz.
† University of Canterbury.
‡ New Zealand Institute for Crop & Food Research Ltd.

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reasons why proteins may not form amyloid fibrils within a heterogeneous mixture, including the kinetics of mixed amyloid fibril formation (*22*) and the presence of chaperone proteins, which reduce the extent of protein denaturation and aggregation through protein−protein interactions (*21*). It has also been observed that the efficiency of seeding fibril formation depends strongly on the sequence similarity (*23*). Thus, to test whether amyloid-like fibrils may be generated in foods, bovine serum albumin (BSA), purified ovalbumin, and egg white were monitored during aggregation using a combination of techniques: the binding of dye molecules that bind amyloid fibrils, X-ray fiber diffraction, and CD spectroscopy.

MATERIALS AND METHODS

Materials. Bovine serum albumin (98% pure, A-7030), ovalbumin (98% pure, A-5503), and other chemicals were obtained from Sigma Chemical Company (St. Louis, MO). Protein concentration was measured by the method of Bradford (*24*). Eggs were obtained from a local supermarket.

Incubation of Protein. Proteins were incubated at various temperatures in buffer containing 50 mM sodium phosphate, 0−200 mM NaCl, 0.5−20 mg mL^{-1} protein, pH 7.0. Aliquots were taken at varying time intervals and placed on ice prior to assay. Ten minute incubations were also carried out using 0.2−0.4 mg mL^{-1} protein in buffer containing 20 mM sodium phosphate buffer, 150 mM NaF, pH 8.0, to mimic the conditions of CD spectroscopy.

Fluorescence Measurements with Thioflavin T. Fluorescence measurements were carried out using a Cary Eclipse Varian fluorescence spectrophotometer using a method based on that of Le Vine (*25*). The assay solution (50 mM Tris-HCl, 100 mM NaCl, 5−20 μM ThT, 20 μg mL^{-1} protein, pH 7.5) was excited at 450 nm with a slit width of 5 nm, and the emission was measured over a range that included the wavelength of 482 nm with a slit width of 10 nm.

Circular Dichroism Spectroscopy. Protein (0.2−0.4 mg/mL) was dialyzed into buffer containing 20 mM sodium phosphate, 150 mM NaF, pH 8.0. CD spectra measurements (Olis DSM-10) were recorded using a 1.0 mm path length quartz cell at a wavelength of 222 nm. Values of ellipticity were expressed in terms of millidegrees, as the exact composition of the egg white was not known.

X-ray Fiber Diffraction. A solution containing 20 mM sodium phosphate, pH 7.0, 200 mM NaCl, and 20 mg/mL BSA was incubated at 85 °C for 5 min in a heat block and allowed to cool over several hours. Five microliters of solution was suspended between the ends of two wax-filled capillaries and allowed to dry in air at room temperature. The sample was aligned in an X-ray beam, and diffraction patterns were collected using a Cu Kα Riguku RU200 rotating anode source (1.5418 Å) and R-AXIS IV image plate X-ray detector. Images were analyzed by Java application "Crystal Clear" software.

RESULTS AND DISCUSSION

Fluorescence of thioflavin T has been widely used as a specific test for the formation of β-structure, including the cross-β structure found in amyloid fibrils (*25*, *26*). Heat denaturation of ovalbumin, as well as crude egg white mixtures and BSA, was associated with an increase in thioflavin T fluorescence, consistent with the formation of amyloid cross-β structure (**Figure 2**). Heat denaturation was also measured using CD spectroscopy, which demonstrated that the structural changes associated with protein denaturation occurred on a similar time scale to the formation of cross-β structure (**Figure 1**). Helical structures have minima at 222 and 208 nm, while β-sheet structure gives minima at 218 nm, and thus an increase in the ellipticity at 222 nm represents a loss of helical structure. There was a good correlation between the increase in thioflavin T fluorescence and the decrease in ellipticity at 222 nm, suggesting that α-structure was being lost as the protein unfolded. Furthermore, calculation of the secondary structure from the spectra for ovalbumin and egg white showed that β-sheet structure was being formed at higher temperatures (**Table 1**), and the ellipticity at 222 nm showed a sigmoidal response to temperature (**Figures 1** and **2**).

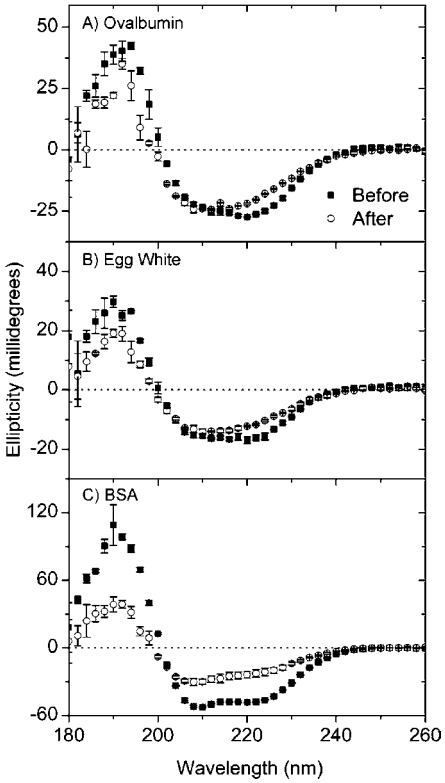

Figure 1. Circular dichroism spectra (±SD) of native (■) and heat denatured (○) ovalbumin (panel **A**), egg white (panel **B**), and BSA (panel **C**). Protein (0.2 mg mL^{-1}) was dialyzed into buffer containing 20 mM sodium phosphate, 150 mM NaF, pH 8.0. Samples were heated at 90 °C for 10 min and then cooled to 20 °C.

Table 1. Secondary Structure Content Was Calculated from CD Spectra (Shown in Figure 1) of Ovalbumin and BSA before and after Heating, Using the CDSSTR Program with Reference Protein Set SP43, as Part of the CDPro Software Package (*39*)

		fraction of secondary structure			
		helix	strand	turn	unordered
ovalbumin	before	0.47	0.12	0.16	0.26
	after	0.42	0.25	0.13	0.21
BSA	before	0.51	0.11	0.12	0.26
	after	0.22	0.26	0.21	0.30

Serum albumin proteins are mostly helical, with no β-sheets (*27*), which is consistent with the secondary structure calculated from the CD spectra before heating (**Table 1**). After heating, BSA lost helical structure, with the concomitant gain of β-sheet structures (**Table 1**), and the ellipticity at 222 nm showed a linear response to temperature (**Figures 1** and **2**). Fluorescence intensity increased at higher temperatures, due to a higher degree of protein denaturation, and followed the pattern of ellipticity loss at 222 nm.

Formation of amyloid fibrils normally follows a lag phase, during which the concentration of intermediate compounds reaches a critical level. However, recent studies have shown that a competing pathway in which semiflexible fibrils are formed rather than rigid long straight fibrils does not involve a nucleation step (*28*). Thioflavin T fluorescence of ovalbumin, crude egg white, and BSA showed an initial rapid increase, with little increase after 10 min (**Figures 3**, **4**, and **5**), which indicates that there is a very minimal lag phase, or that the increase in fluorescence

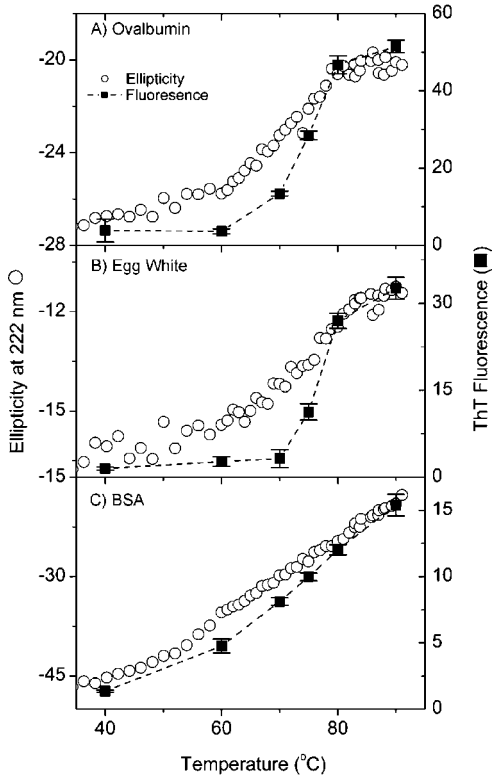

Figure 2. Changes in the ellipticity at 222 nm (○) and fluorescence intensity (±SD) with ThT (■) during heat denaturation of ovalbumin (panel **A**), egg white (panel **B**), and BSA (panel **C**). Protein (0.2 mg mL^{-1}) was dialyzed into buffer containing 20 mM sodium phosphate, 150 mM NaF, pH 8.0. Samples with incubated at the indicated temperatures for 10 min before being assayed for ThT fluorescence at 482 nm. Ellipticity at 222 nm was recorded at 2 °C increments up to 60 °C and then 1 °C increments up to 90 °C using a 1.0 mm path length quartz cell.

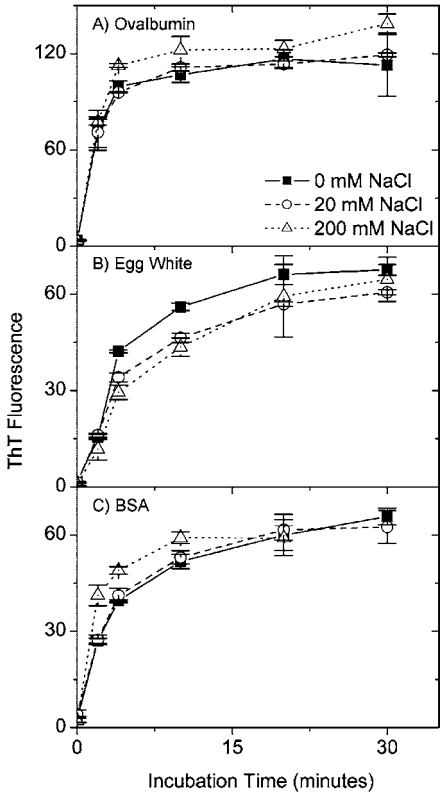

Figure 3. A 20 mg mL^{-1} solution of ovalbumin (panel **A**), egg white (panel **B**), and BSA (panel **C**) were incubated at 80 °C in buffer containing 20 mM sodium phosphate buffer, pH 7.5, and 0 (■), 20 (○), or 200 (△) mM NaCl. Aliquots were taken at the indicated time periods and placed on ice until measurement for ThT fluorescence at 482 nm (±SD).

was a non-nucleated event. Previous work using transmission electron microscopy has suggested that, at low pH, ovalbumin and BSA form semiflexible amyloid fibrils (*29*), and the lack of a lag phase in this study shows that this is likely to occur at physiological pH as well.

Several factors have been shown to influence the extent of amyloid fibril formation, including the ionic concentration, protein concentration, and the temperature (*30*, *31*). Varying the ionic concentration did not appear to influence the increase in thioflavin T fluorescence for ovalbumin, crude egg white, or BSA (**Figure 3**). Increasing the protein concentration increased the extent of thioflavin T fluorescence for crude egg white and BSA, but did not have as much influence for ovalbumin (**Figure 4**). The largest factor influencing thioflavin T fluorescence was temperature, with much higher fluorescence at higher temperatures for ovalbumin, crude egg white, and BSA (**Figure 5**).

A comparison of the denaturation of ovalbumin and crude egg white showed that the crude egg white had a final thioflavin fluorescence which was around 60% of ovalbumin. This is consistent with ovalbumin, which constitutes 54% of the egg white, being the only protein in the mixture to form a fluorescent complex with thioflavin T. At lower protein concentrations (**Figure 4**) and lower temperatures (**Figure 5**), the extent of thioflavin fluorescence was lower for the egg white mixture than for the purified ovalbumin, suggesting that interactions with other proteins may reduce the degree of ordered structure formed during aggregation and favor amorphous aggregation.

Examination of the heated protein mixtures using transmission electron microscopy showed that nearly all of the protein was present as amorphous aggregates, rather than having a high degree of ordered structures (data not shown). The absolute thioflavin T fluorescence shown by these protein samples is lower than that observed for other fibril samples (*25*), suggesting that only a small fraction of protein is involved in fibril formation, and this is also reflected in the only slight increase in β-sheet content, as shown by CD spectroscopy.

While thioflavin T has traditionally been used as an indicator for the presence of amyloid, inconsistent results can be observed due to nonspecific dye binding or changes in the spectral properties of the dye with different buffers and proteins (*26*). A more definitive method for amyloid fibril formation is the X-ray fiber diffraction pattern (*13*). Amyloid fibrils typically show dominant reflections at 4.7−4.8 Å, corresponding to the spacing between adjacent β-strands in the direction of the fibril axis, and at approximately 10 Å, corresponding to the face-to-face separation of the β-sheets (*13*).

To determine if the increase in thioflavin T fluorescence for BSA was indeed caused by formation of amyloid cross-β structure, the X-ray fiber diffraction pattern of denatured protein was investigated (**Figure 6**). This pattern showed two distinctive rings at 4.6 and 9.7 Å, which is indicative of the cross-β structure, and suggests that the aggregated BSA does indeed have an amyloid-like structure.

In conclusion, heat denaturation of proteins is a common process in food processing and can result in protein aggregation. CD spectroscopy was used to monitor the changes in protein structure when samples of bovine serum albumin and both pure ovalbumin and crude egg white were denatured by heating. Changes in the CD spectra suggested that β-sheet structure was being formed at high temperatures, and the changes in ellipticity

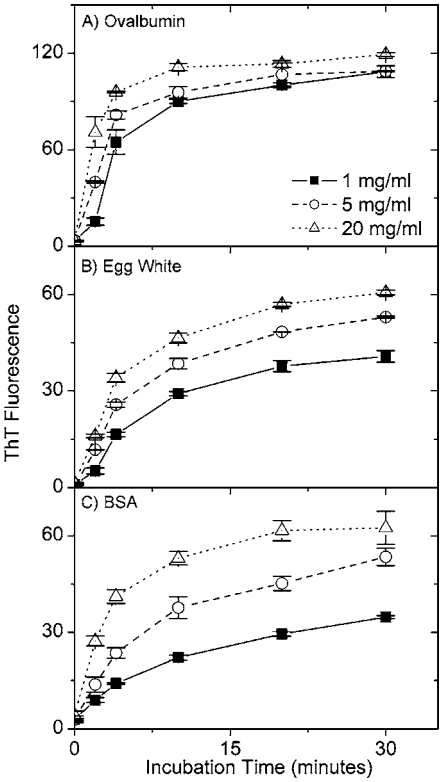

Figure 4. A 1 (■), 5 (○), or 20 (△) mg mL^{-1} solution of ovalbumin (panel **A**), egg white (panel **B**), and BSA (panel **C**) were incubated at 80 °C in buffer containing 20 mM sodium phosphate buffer, pH 7.5, 200 mM NaCl. Aliquots were taken at the indicated time periods and placed on ice until measurement for ThT fluorescence at 482 nm (±SD).

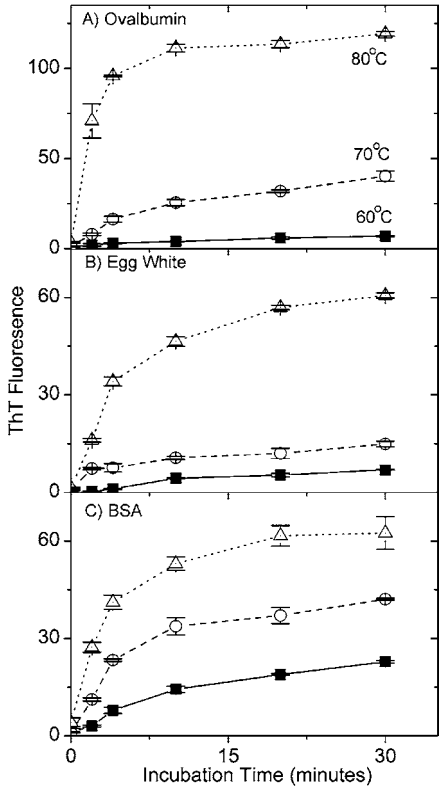

Figure 5. A 20 mg mL^{-1} solution of ovalbumin (panel **A**), egg white (panel **B**), and BSA (panel **C**) were incubated at 60 °C (■), 70 °C (○), or 80 °C (△) in buffer containing 20 mM sodium phosphate buffer, pH 7.5, 200 mM NaCl. Aliquots were taken at the indicated time periods and placed on ice until measurement for ThT fluorescence at 482 nm (±SD).

at 222 nm correlated to an increase in fluorescence with thioflavin T, which is an amyloid specific dye. It was found that the extent of thioflavin T fluorescence was highly dependent on temperature, with lesser dependence on protein concentration and ionic strength, and that the increase in fluorescence did not show the lag phase that is often associated with fibril formation. As a further test of amyloid fibril formation, X-ray fiber diffraction patterns of denatured bovine serum albumin proteins were consistent with those of amyloid fibrils.

The results described here suggest that protein aggregates seen during food processing may be amyloid-like in nature, although it is uncertain whether they resemble the traditional rigid long-straight fibrils, or the semi-flexible (worm-like) fibrils (*28*). Fibril-like structures have been reported in mixtures of whey proteins (*32*), particularly in the formation of aggregate gels. Other work has also suggested that fibril-like structures may assist in gel formation at low protein concentrations (*33*).

Recent reports have shown that naturally occurring protein fibrils can exert amyloid accelerating properties in mice (*34*). However, increasing evidence suggests that amyloid toxicity is associated with intermediates in the folding pathway (*35*, *36*) and, further, that it is restricted to specific sequences (*23*). The pathological process of amyloidogenesis usually requires proteolysis of precursor proteins (*37*), with examples including Alzhemier's disease, secondary systemic amyloidosis, and type II diabetes. This is backed up by the growing number of examples of amyloids in nature, such as spiders webs (*4*), moth silk (*5*), and melanosomes of humans (*10*), which are not associated with disease (*38*), and so it is unlikely that amyloid-like compounds formed during food processing present a food safety hazard.

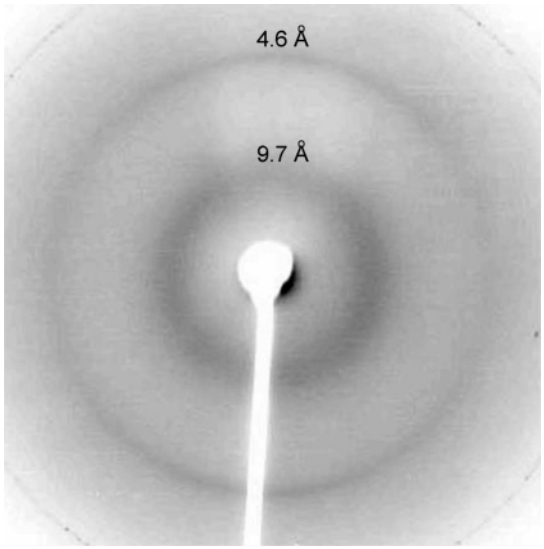

Figure 6. X-ray fiber diffraction pattern from BSA incubated as described in the Materials and Methods section, showing the positions of reflections at 4.6 and 9.7 Å.

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