

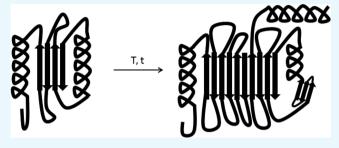


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Ultrasonic Characterization of Amyloid-Like Ovalbumin Aggregation

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ABSTRACT: Thermal processing conditions, pH, and salt content affect the formation of egg white ovalbumin amyloid, which was investigated using high-precision measurements of ultrasonic velocity and attenuation. These were related to fluorescence and particle size measurements. Fluorescence changes indicated the formation of amyloid-like aggregates that was enhanced by increasing time-temperature treatments. The ultrasonic velocity of ovalbumin after heating at neutral pH (60 min at 70 or 80 °C) was lower than that of unheated ovalbumin, whereas the attenuation was higher. The



decrease in the velocity represents increased compressibility associated with a change in the compactness of the protein, whereas changes in attenuation are due to protein conformational changes. Heating ramp studies revealed transitions at approximately 58 and 73 °C. During heating at a constant temperature, the ultrasonic velocity decreased slowly with increasing heating time, indicating an increase in ovalbumin compressibility. It is suggested that the obtained amyloid-like ovalbumin aggregates contain a compact core surrounded by loosely packed protein segments.

INTRODUCTION

The term amyloids originally referred to filamentous protein deposits in the human body, with structures that are linked to several diseases. However, several food proteins can also form amyloid-like fibrils, such as lysozyme, 2,3 ovalbumin, 4,5 β -lactoglobulin, 6,7 bovine serum albumin, 8,9 α -lactalbumin, 10,11 and soy glycinin. 12,13 The molecular structure characterizing amyloid fibrils is the cross- β motif consisting of β -strands that are stacked in the direction perpendicular to the long axis of the fibril, linked by interstrand hydrogen bonds. 14 The presence of amyloid-like aggregates is typically indicated by staining with dyes such as Congo red or thioflavin T (ThT).

The focus of this work is the formation of amyloid-like aggregates from ovalbumin. Ovalbumin, which constitutes about half of the proteins in avian egg white, is a glycophosphoprotein with a molecular weight of 45 kDa and an isoelectric point of 4.5. The sequence of 385 amino acids contains six cysteine residues, of which two are involved in one disulfide bond and the rest occur as free sulfhydryl groups. 15-17 The denaturation temperature of ovalbumin at neutral pH is about 78 °C. During storage and mild heating under alkaline conditions, the more heat-stable S-ovalbumin with a denaturation temperature of 86 °C is formed from native ovalbumin. 18,19

Amyloid-like aggregates can be formed from ovalbumin during heating under both acidic and neutral pH conditions.^{4,5} Process conditions, ionic strength, and pH are typical parameters that affect amyloid formation. 4,20-22 The Nterminal region and the disulfide bond of ovalbumin also strongly influence the morphology of aggregates formed upon

heating. 5,23 Upon reduction of the disulfide bond, long straight fibrils are formed during heating at neutral pH, whereas semiflexibile fibrils are usually observed after heating intact ovalbumin. 5,24,25 The degree of branching increased with increasing ionic strength.²⁴ During the amyloid formation of ovalbumin, the β -sheet content typically increases. 5,23

Compressibility studies are frequently used to study conformational transitions of globular proteins. 26-29 Furthermore, they provide valuable insights into protein hydration and intraglobular packing of protein aggregates.³⁰ The compressibility of proteins in solution can be determined by measuring both the ultrasonic velocity and density, with the protein's compressibility composed of a hydration compressibility and an intrinsic compressibility. 31,32 The hydration compressibility is due to the change in the solvent compressibility as water molecules interact with the solvent-accessible groups of the proteins. Because water in the hydration shell is less compressible than free bulk water, the hydration compressibility is usually negative. ^{29,31,32} The role of water in protein functionality cannot be understated, 30,33 so techniques that assess changes in the restriction of water molecules in the vicinity of proteins provide insights into the relationship between the structure and behavior of proteins. 28,33,34 The intrinsic compressibility is due to imperfect packing of the polypeptide chain within the solvent-inaccessible interior of the protein. It provides a positive contribution to the protein's

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overall compressibility. For liquids, the adiabatic compressibility coefficient (βs) can be calculated from the Laplace equation on the basis of density (ρ) and ultrasound velocity (u) measurements³⁴

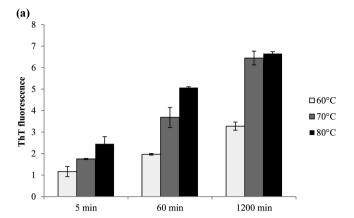
$$\beta s = 1/(\rho \cdot u^2) \tag{1}$$

The principles of high-precision compressibility measurements with ultrasound as well as their relation to the protein structure have been recently reviewed by Zhang et al.35 Here, only selected aspects related to fibril formation will be highlighted. The volumetric changes upon the fibril formation seem to depend on the protein. Amyloid fibrils from β_2 -microglobulin and from a disulfide-deficient variant of hen egg white lysozyme are more voluminous than the native structure, whereas a compaction was reported upon insulin fibril formation. 39,40 These disparities in volumetric changes upon the fibril formation of proteins likely reflect the intrinsic feature of polymorphism of amyloid-like fibrils.³⁶ Interestingly, in contrast to the voluminous amyloid fibrils formed from β_2 -microglobulin, fibrils formed from the amyloidogenic core peptide (K3) of β_2 -microglobulin were highly compact. On the basis of these results, Lee et al.³⁶ suggested that the amyloid fibrils of β_2 -microglobulin consist of a tightly packed core structure surrounded by solvent-inaccessible areas with a loose packing, rendering the amyloid fibrils compressible. Clearly, more data are needed to comprehensively elucidate the trends in the volumetric changes upon the amyloid fibril formation.³

In this research, we varied several factors that induce amyloid-like formation in proteins (pH, salt, and thermal processing) to generate systems with varying degrees of amyloid-like aggregation in ovalbumin, the major protein in egg white. The effect of disulfide reduction and redox agents has been discussed elsewhere.⁴¹ The compressibility and hydration of these systems were determined from ultrasonic measurements made at room temperature after prior formation of amyloid-like aggregates at a high temperature as well as in real time during amyloid formation at several temperatures. To relate our understanding on the ovalbumin structural changes derived from such measurements to those from conventional amyloidogenesis assays, we interpreted the high-precision ultrasonic measurements of velocity and attenuation in the context of fluorescence and dynamic light scattering (DLS) measurements on the same systems.

■ RESULTS AND DISCUSSION

Heating of Ovalbumin at Neutral pH. Fluorescence Measurements and Particle Size. Ovalbumin (2.0% w/w) was heated in the presence or absence of NaCl at different temperatures for up to 1200 min. Figure 1a,b shows the ThT fluorescence and surface hydrophobicity of the samples after cooling to room temperature, respectively. The higher ThT fluorescence after heating suggests that ovalbumin forms cross- β structures typical for the amyloid-like aggregation when heated at neutral pH, which is in agreement with literature reports. 21,22 The ThT fluorescence increased with heating time and heating temperature. Similar fluorescence values were recorded after overnight heating (1200 min) at 70 and 80 °C. The presence of salt did not affect the ThT fluorescence after heating at 60 and 70 °C for 60 min (Figure 1a and Table 1). Samples containing salt heated at 70 °C for 1200 min and at 80 °C for 60 min were highly viscous and turbid and, hence, could not be analyzed.



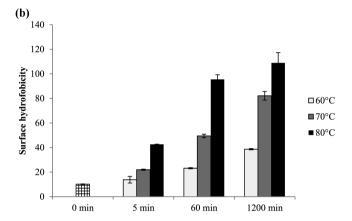


Figure 1. (a) ThT fluorescence of ovalbumin (2.0% w/w, pH 7.0) in the absence of salt after heating at 60, 70, and 80 °C for 5, 60, and 1200 min. The fluorescence data are normalized to the unheated sample. Error bars indicate the range of duplicate sample preparations. (b) Surface hydrophobicity, calculated from 1-anilino-8-naphthalene sulfonate (ANS) fluorescence measurements of ovalbumin (2.0% w/w, pH 7.0) in the absence of salt before and after heating at 60, 70, and 80 °C for 5, 60, and 1200 min. Error bars indicate the range of duplicate sample preparations.

The surface hydrophobicity calculated from fluorescence measurements in the presence of the fluorescent probe ANS increased with heating time and temperature (Figure 1b). Conformational changes during the sample treatment evidently result in the exposure of previously buried hydrophobic moieties. The temperature-induced changes in surface hydrophobicity were unaffected by the presence of salt when heating at 60 and 70 °C for 60 min (Table 1).

Even though clear changes in the surface hydrophobicity and ThT fluorescence were observed after prolonged heating of ovalbumin at 60 °C and neutral pH, the particle size was hardly affected (Table 1). Heating at either 70 or 80 °C yielded particles of increased size, but even after 1200 min of heating, the particle size was still relatively small (about 10 nm). Similar sizes were observed for heated solutions of ovalbumin by Weijers et al. Significantly bigger particles were formed when heating was performed in the presence of salt. This indicates that the charges on ovalbumin at neutral pH restrict its aggregation during heating. By ionic shielding of the charges, bigger particles form under similar heating conditions. Ovalbumin heated in the presence of salt at 70 °C for 1200 min and at 80 °C for 60 min was too viscous for analyses, which is likely due to the presence of large branched aggregates.

Table 1. Normalized ThT Fluorescence, Surface Hydrophobicity, and Hydrodynamic Radius (nm) of Ovalbumin (2.0% w/w) with and without Salt at pHs 2.0, 7.0, and 9.0 before and after Heating at 60, 70, and 80 °C for 60 and 1200 min

| sample | ThT fluorescence ^a | surface hydrophobicity ^a | hydrodynamic radius ^a |
|-------------------------|-------------------------------|-------------------------------------|----------------------------------|
| oH 7.0—no salt | | | |
| unheated | 1 (-) | 10.2 (0.2) | 3.0 (0.0) |
| 60 °C—60 min | 2.0 (0.0) | 23.1 (0.5) | 2.9 (0.6) |
| 60 °C—1200 min | 3.3 (0.2) | 39.6 (0.5) | 3.6 (0.1) |
| 80 °C—60 min | 5.1 (0.0) | 95.4 (3.8) | 7.0 (0.5) |
| 80 °C—1200 min | 6.7 (0.1) | 108.9 (8.5) | 7.0 (0.5) |
| H 7.0—salt ^b | | | |
| unheated | 1 (-) | 11.0 (1.4) | 3.0 (0.0) |
| 60 °C—60 min | 1.9 (0.1) | 24.2 (0.3) | 3.3 (0.2) |
| 60 °C—1200 min | 3.3 (0.3) | 40.0 (3.0) | 21.7 (0.9) |
| 70 °C—60 min | 3.7 (0.3) | 48.6 (2.0) | 48.9 (1.0) |
| oH 2.0—no salt | | | |
| unheated | 1 (-) | 72.8 (1.9) | 4.2 (0.4) |
| 60 °C—60 min | 2.8 (0.6) | 108.7 (0.1) | 7.3 (1.6) |
| 60 °C—1200 min | 3.4 (0.2) | 107.6 (8.0) | 8.9 (3.4) |
| 80 °C—60 min | 2.9 (0.3) | 114.9 (29.0) | 10.3 (2.6) |
| 80 °C—1200 min | 2.8 (0.3) | 115.8 (24.0) | 15.8 (1.8) |
| oH 2.0—salt | | | |
| unheated | 1 (-) | 70.3 (4.0) | 4.5 (1.0) |
| 60 °C—60 min | 2.5 (0.3) | 111.6 (4.2) | 10.3 (0.1) |
| 60 °C—1200 min | 2.8 (0.4) | 125.8 (7.3) | 17.2 (0.9) |
| 70 °C—60 min | 2.2 (0.0) | 95.2 (6.6) | 16.9 (4.6) |
| 70 °C—1200 min | 2.4 (0.0) | 101.3 (0.2) | 16.7 (1.8) |
| 80 °C—60 min | 2.4 (0.1) | 96.1 (15.1) | 19.4 (1.5) |
| oH 9.0—no salt | | | |
| unheated | 1 (-) | 11.0 (0.5) | 2.8 (0.1) |
| 60 °C—60 min | 1.5 (0.1) | 31.6 (2.4) | 2.8 (0.1) |
| 60 °C—1200 min | 2.2 (0.2) | 46.0 (3.2) | 3.1 (0.2) |
| 80 °C—60 min | 3.8 (0.2) | 95.4 (13.5) | 4.5 (0.1) |
| 80 °C—1200 min | 4.9 (0.4) | 106.3 (10.8) | 4.8 (0.0) |
| oH 9.0—salt | | | |
| unheated | 1 (-) | 11.3 (2.1) | 3.1 (0.1) |
| 60 °C—60 min | 2.0 (0.0) | 44.2 (1.1) | 3.3 (0.2) |
| 60 °C—1200 min | 2.6 (0.0) | 48.6 (0.2) | 3.4 (0.1) |
| 70 °C—60 min | 2.7 (0.3) | 59.3 (0.6) | 4.1 (0.5) |
| 70 °C—1200 min | 4.6 (0.1) | 95.1 (0.4) | 10.4 (1.9) |
| 80 °C—60 min | 4.9 (0.1) | 120.0 (7.3) | 13.5 (0.9) |

[&]quot;Each sample was prepared twice and then analyzed multiple times depending on the measurement technique (duplicate for ThT measurements, single for the surface hydrophobicity and fivefold for the particle size determination). In this table, the range of duplicate sample preparations is reported in parentheses (i.e., the difference between the averages of the one- to fivefold measurements (depending on the measurement technique) of both sample preparations). ^bSamples containing salt heated at 70 °C for 1200 min and at 80 °C for 60 min were highly viscous and turbid and, hence, could not be analyzed.

Table 2. Ultrasonic Velocity (m/s) of Ovalbumin (2.0% w/w, pH 7.0) with and without Salt before and after Heating at 60, 70, and 80 °C for 5, 60, and 1200 min

| | heating time ^a | | | |
|--------------|---------------------------|----------------|----------------|----------------|
| sample | 0 min | 5 min | 60 min | 1200 min |
| without salt | | | | |
| 60 °C | 1501.85 (0.02) | 1501.85 (0.02) | 1501.85 (0.01) | 1501.86 (0.01) |
| 70 °C | 1501.86 (0.03) | 1501.83 (0.01) | 1501.80 (0.00) | 1501.83 (0.00) |
| 80 °C | 1501.84 (0.02) | 1501.81 (0.01) | 1501.78 (0.02) | 1501.87 (0.01) |
| with salt | | | | |
| 60 °C | 1503.48 (0.03) | | 1503.46 (0.02) | 1503.45 (0.03) |
| 70 °C | 1503.53 (0.02) | | 1503.41 (0.06) | |

[&]quot;Each sample was prepared twice, and both sample preparations were then analyzed at least six times. The standard deviation of the ultrasonic velocity for each sample preparation was typically below 0.03 m/s. In this table, the range of duplicate sample preparations is reported in parentheses (i.e., the difference between the averages of the six or more measurements of both sample preparations).

Studies on the effect of ionic strength on the morphology of aggregates showed that flexible ovalbumin aggregates with very few branching points are typically formed at low ionic strengths, whereas more densely branched structures form at high ionic strengths. ^{25,42}

Ultrasound Properties. The selected experimental conditions generated a range of changes in ovalbumin conformation and size. We next examined how such structural changes altered the ultrasonic properties of the protein solutions. The ultrasonic velocity of ovalbumin with and without salt remained unaffected by heat treatment at 60 °C, whereas a small but significant increase in the attenuation was observed when heating without salt for at least 60 min at this temperature (Tables 2 and 3). The lack of thermal effect on the ultrasonic

Table 3. Ultrasonic Attenuation $(10^{-14} \text{ s}^2/\text{m})$ of Ovalbumin (2.0% w/w, pH 7.0) with and without Salt before and after Heating at 60, 70, and 80 °C for 5, 60, and 1200 min

| | heating time ^a | | | |
|--------------|---------------------------|-------------|-------------|-------------|
| sample | 0 min | 5 min | 60 min | 1200 min |
| without salt | | | | |
| 60 °C | 2.04 (0.03) | 2.05 (0.06) | 2.15 (0.01) | 2.19 (0.01) |
| 70 °C | 2.08 (0.05) | 2.15 (0.06) | 2.28 (0.07) | 2.48 (0.10) |
| 80 °C | 2.11 (0.03) | 2.25 (0.02) | 2.63 (0.06) | 2.66 (0.00) |
| with salt | | | | |
| 60 °C | 2.18 (0.03) | | 2.17 (0.02) | 2.2 (0.07) |
| 70 °C | 2.00 (0.04) | | 2.21 (0.02) | |

"Each sample was prepared twice, and both sample preparations were then analyzed at least six times. The standard deviation on the ultrasonic attenuation for each sample preparation was typically below $0.05 \times 10^{-14}~\rm s^2/m$. In this table, the range of duplicate sample preparations is reported in parentheses (i.e., the difference between the averages of the six or more measurements of both sample preparations).

velocity of the ovalbumin solution indicates that the adiabatic compressibility of the protein was unaffected by this low-temperature heat treatment (eq 1). This suggests that either the conformational changes induced by the heat treatment at 60 °C, as evinced by fluorescence measurements, are too small to be detected with ultrasound velocity measurements, or several changes induced by heating have opposite effects on the ultrasonic velocity and cancel each other out, as has been observed for thermally induced changes in other proteins.²⁹

Because salt clearly induced the growth of particles (Table 1), particle size does not appear to have a strong effect on the ultrasonic velocity and, thus, on the adiabatic compressibility. Any aggregation would affect the compressibility by decreasing the hydration contribution due to increased protein—protein interactions at the expense of protein—water interactions. A possible explanation for the lack of effect lies in the type of aggregates. It has been reported that ovalbumin aggregates formed after heating at neutral pH at rather low salt concentrations are fibrillar. Hence, the area of contact between aggregating molecules would be smaller than the overall surface area exposed to water.

Heat treatment at 70 and 80 $^{\circ}$ C affects both the ultrasonic velocity and the attenuation (Tables 2 and 3). After heating for 60 min at 70 and 80 $^{\circ}$ C, the velocity was significantly lower than that of the unheated samples. However, after 1200 min of heating at 80 $^{\circ}$ C, the ultrasonic velocity was actually greater than that after heating for 60 min. The decrease in the velocity

after heating for 60 min indicates that the protein molecules are more compressible.

A significantly higher attenuation was observed after heating the sample for 60 min at 70 $^{\circ}$ C than for the unheated sample, and this increased further with prolonged heating (Table 3). The attenuation was already significantly higher than that of the unheated sample after 5 min of heating at 80 $^{\circ}$ C, increased further with increasing the heating time up to 60 min, and leveled off with further heating.

On the basis of the various techniques, a picture of ovalbumin's thermally induced conformational changes was constructed and is shown graphically in Figure 2. Heating at 60

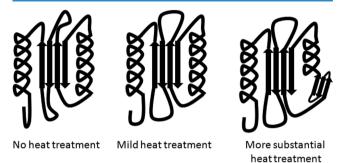




Figure 2. Schematic representation of ovalbumin's thermally induced conformational changes. A distinction is made between different levels of heat treatment.

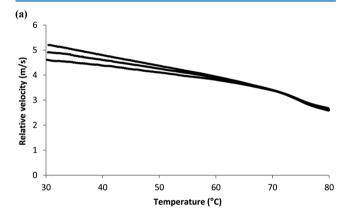
°C and neutral pH for at least 60 min induces structural changes (ThT and ANS fluorescence), but this altered structure does not lead to an increase in size (DLS) unless charge repulsion is suppressed, in which case long-term heating does increase the aggregate size. The compressibility of these molecules is unaltered (velocity), but the changes in conformation brought about by the thermal treatment does cause a greater dissipation of the acoustic energy (increased attenuation).

With an increase in temperature, more substantial changes in the ovalbumin structure take place (Figure 2). A loosening of protein structure allows the hydrated ovalbumin to become more compressible. As the molecular structural changes associated with enhanced surface hydrophobicity and ThT fluorescence tend to decrease compressibility (due to a less compressible hydration shell and compact cross- β structures), it is the overall molecular structure that becomes less compact. These heat-induced protein structures likely contain a core composed of compact, cross- β structures, surrounded by loosely packed areas, as was suggested for β_2 -microglobulin fibrils. Attenuation changes are in line with the changes observed by ThT and ANS fluorescence rather than with the changes in size (as measured with DLS), pointing to the substantial conformational changes as temperature is increased.

Greater viscous losses associated with an opening up of ovalbumin's structure is one potent contributor to the increased attenuation. As heating at these elevated temperatures is continued for extensive periods, a tightening up of the structure occurs.

Real-Time Analysis. The ultrasonic properties of the above samples depended not only on the changes that occur during heat treatment but also on the folding during cooling to room temperature. To learn more about changes during heating at the selected temperatures, ovalbumin samples were subjected to a thermal ramp (heating from 30 to 60, 70, or 80 °C at 0.3 °C/min) followed by a holding phase of 120 min at the final temperature. During heating, the ultrasonic velocity of both ovalbumin solution and the reference, water, increased with increasing temperature up to about 70 °C and decreased with further temperature increase. To investigate the effect of temperature on the proteins alone, the ultrasonic profiles of the reference sample (water) were subtracted from those of the ovalbumin solution. 43

Figure 3a shows the difference in velocity between an ovalbumin solution at pH 7.0 and the reference, defined as the relative velocity, when heating from 30 to 80 °C. In agreement with the studies on whey protein⁴³ and α -lactalbumin,⁴⁴ a



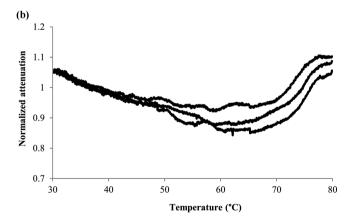


Figure 3. (a) Relative ultrasonic velocity of ovalbumin (2.0% w/w, pH 7.0) during heating from 30 to 80 °C at 0.3 °C/min. The relative ultrasonic velocity is the difference between the velocity of the ovalbumin solution and the velocity of the reference, water. Three representative curves are shown. (b) Normalized ultrasonic attenuation of ovalbumin (2.0% w/w, pH 7.0) during heating from 30 to 80 °C at 0.3 °C/min. The attenuation is normalized compared with the ultrasonic attenuation value at 30 °C. Three representative curves are shown.

decrease in the relative velocity with increasing temperature was observed for ovalbumin solutions. The decrease in the relative velocity with increasing temperature in this study was highly linear to approximately 58 °C, at which point the decrease in the relative velocity accelerated, and this acceleration was even more at approximately 73 °C. Similar temperature inflexion points were observed for normalized attenuation (Figure 3b). Therefore, the changes in the protein structure that affected the fluorescence as temperature was raised above 58 °C, but were accelerated considerably just above 70 °C, concurred with the enhanced reductions in velocity 45 and increases in attenuation 46 brought about by the thermal treatments (Table 1 and Figure 1).

Following the heating ramp, the samples were kept at the final temperature (60, 70, and 80 °C) for 120 min. In line with expectations from the temperature ramp results of Figure 3, holding at 70 °C did lead to changes in the ultrasonic properties. The ultrasonic velocity decreased continuously as a function of time (total decrease: 0.17 ± 0.02 m/s), and the attenuation increased (total increase: $1.8 \pm 0.5 \times 10^{-15}$ s²/m). It is interesting to point out that, although the decrease in velocity was most pronounced during the first 45 min of holding, the decrease occurred over the entire 120 min (Figure 4). The conformational changes at this temperature were thus

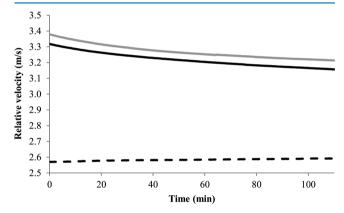


Figure 4. Relative ultrasonic velocity of ovalbumin (2.0% w/w) at pH 2.0 (black, dashed line), pH 7.0 (black, full line), and pH 9.0 (gray, full line) during isothermal heat exposure at 70 °C for 120 min. The relative ultrasonic velocity is the difference between the velocity of the ovalbumin solution and the velocity of the reference, water, with the same amount of acid/alkali as the sample.

rather slow. This is in agreement with the amyloid-like aggregation, which is typically slow. The decreasing relative velocity upon holding at 70 °C indicates that the compressibility increased with a prolonged heat exposure. The increasing formation of cross- β structures in the core of amyloid-like aggregates likely goes hand in hand with a greater formation of loosely packed areas surrounding that core. This seems reasonable when considering that only part of the amino acid sequence of ovalbumin is involved in the cross- β structures of the amyloid core. The increased attenuation during holding at 70 °C is consistent with the conformational changes.

Both the ultrasonic velocity and attenuation remained constant during holding at 80 °C (results not shown). Apparently, no changes affecting the ultrasonic properties occurred during holding at 80 °C. The lack of structural changes when holding at 80 °C is because most of the ovalbumin are already unfolded before the start of the holding

phase, as its denaturation midpoint temperature is 78 °C.¹⁶ The instrument's rather low heating rate (to ensure highly regulated temperatures) also exposes samples to relatively high temperatures for a long time before the start of the holding phase (e.g., heating from 70 to 80 °C at 0.3 °C/min takes more than 30 min).

The changes in velocity and attenuation during holding at 60 °C were similar to those at 70 °C, but less pronounced. At 60 °C, the ultrasonic velocity decreased continuously as a function of time (total decrease: 0.03 ± 0.01 m/s) and the attenuation increased (total increase: $0.6 \pm 0.09 \times 10^{-15}$ s²/m). Smaller changes in the ultrasonic properties during holding at 60 °C are consistent with the changes in fluorescence. Although no significant effect on the ultrasonic properties was observed when heating at 60 °C was followed by cooling (Tables 2 and 3), a small, but clear, effect was observed here, as the ovalbumin solutions were maintained at 60 °C, pointing to the significance of 58 °C as a critical transition temperature point for ovalbumin.

Heating of Ovalbumin at Acidic and Alkaline pH. Because molecular charges have pronounced effects on protein aggregation at different temperatures, ^{15,33} we investigated how changes in ovalbumin's charge density induced changes in its ultrasonic properties.

Fluorescence Measurements and Particle Size. Just as for ovalbumin at neutral pH, the ThT fluorescence at pH 9.0 increased with heating time and temperature (Table 1), but it was less pronounced. The ThT fluorescence was also higher after heating at pH 2.0; however, in contrast to the changes at pHs 7.0 and 9.0, the ThT fluorescence did not increase with temperature.

Regardless of pH, surface hydrophobicity increased for all heating conditions tested. It is of note that at pH 2.0 the surface hydrophobicity of unheated ovalbumin was already much higher than that at pHs 7.0 and 9.0, showing that acidification induced conformational changes, thus exposing hydrophobic moieties. In contrast to the samples at pHs 7.0 and 9.0, those at pH 2.0 exhibited similar surface hydrophobicity, irrespective of the duration of heating.

The effect of heating on particle size (Table 1) depended on both pH and salt. Similar to that at neutral pH, the particle size did not increase at pH 9.0 when ovalbumin was heated at 60 °C without salt. In the presence of salt, the increase in the particle size of the ovalbumin molecules, which was already observed at 60 °C at neutral pH, was arrested until higher temperatures were attained at pH 9.0. The particle size of the aggregates formed at 70 °C in the presence of salt was also much smaller at pH 9.0, as the electrostatic repulsion of the higher charge density on the molecules hinders protein aggregation. ⁴² Charge density effects were not evident at pH 2.0 although bigger particles were formed when salt was present. This enhanced aggregation is probably related to the low-temperature acidinduced conformational changes with the exposed hydrophobic moieties driving ovalbumin aggregation.

Ultrasound Properties. (Tables 4 and 5 show the ultrasonic velocity and attenuation of ovalbumin at pHs 2.0 and 9.0 before and after heat treatment at different temperatures. As several papers have dealt with the effect of pH on the ultrasonic properties of unheated proteins, ^{27,49–51} we only discuss the effect of heat treatment at different pHs.

An increase in the velocity with prolonged heating at 60 °C in the presence of salt and at 80 °C without salt (pH 9.0) suggests a decrease in the compressibility (eq 1). This decrease

Table 4. Ultrasonic Velocity (m/s) of Ovalbumin (2.0% w/w, pHs 2.0 and 9.0) with and without Salt before and after Heating at 60, 70, and 80 °C for 60 and 1200 min

| | heating time ^a | | |
|----------------|---------------------------|----------------|----------------|
| sample | 0 min | 60 min | 1200 min |
| pH 9.0—no salt | | | |
| 60 °C | 1501.92 (0.00) | 1501.94 (0.01) | 1501.96 (0.02) |
| 80 °C | 1501.89 (0.02) | 1501.93 (0.05) | 1502.03 (0.02) |
| pH 9.0—salt | | | |
| 60 °C | 1503.21 (0.03) | 1503.16 (0.03) | 1503.24 (0.00) |
| 70 °C | 1503.52 (0.05) | 1503.49 (0.01) | 1503.52 (0.08) |
| 80 °C | 1503.52 (0.05) | 1503.43 (0.05) | |
| pH 2.0—no salt | | | |
| 60 °C | 1502.07 (0.14) | 1501.93 (0.08) | 1501.98 (0.12) |
| 80 °C | 1502.20 (0.03) | 1502.11 (0.06) | 1502.55 (0.11) |
| pH 2.0—salt | | | |
| 60 °C | 1503.19 (0.03) | 1503.12 (0.00) | 1503.19 (0.00) |
| 70 °C | 1503.78 (0.00) | 1503.66 (0.04) | 1503.79 (0.00) |
| 80 °C | 1503.78 (0.00) | 1503.74 (0.01) | |

^aTypical standard deviations and representation mode as in Table 2.

Table 5. Ultrasonic Attenuation $(10^{-14}~\rm s^2/m)$ of Ovalbumin (2.0% w/w, pHs 2.0 and 9.0) with and without Salt before and after Heating at 60 and 70 °C for 60 and 1200 min

| | heating time ^a | | |
|----------------|---------------------------|-------------|-------------|
| | | | |
| sample | 0 min | 60 min | 1200 min |
| pH 9.0—no salt | | | |
| 60 °C | 2.03 (0.02) | 2.20 (0.07) | 2.18 (0.07) |
| 80 °C | 2.14 (0.09) | 2.51 (0.08) | 2.54 (0.07) |
| pH 9.0—salt | | | |
| 60 °C | 2.05 (0.02) | 2.14 (0.01) | 2.11 (0.03) |
| 70 °C | 2.02 (0.03) | 2.21 (0.03) | 2.37 (0.01) |
| 80 °C | 2.02 (0.03) | 2.59 (0.03) | |
| pH 2.0—no salt | | | |
| 60 °C | 2.29 (0.11) | 2.59 (0.04) | 2.66 (0.08) |
| 80 °C | 2.23 (0.02) | 2.66 (0.01) | 2.60 (0.07) |
| pH 2.0—salt | | | |
| 60 °C | 2.20 (0.01) | 2.5 (0.02) | 2.55 (0.08) |
| 70 °C | 2.40 (0.01) | 2.66 (0.03) | 2.62 (0.03) |
| 80 °C | 2.40 (0.01) | 2.73 (0.03) | |

^aTypical standard deviations and representation mode as in Table 2.

in compressibility was greater at pH 9.0 than at pH 7.0. There were no obvious differences in the surface hydrophobicity and ThT fluorescence that explained such differences (Table 1). Either the hydration contribution is higher (more negative) at pH 9 or the intrinsic contribution is lower. When heated at a mild temperature under alkaline conditions, ovalbumin is converted to the more thermostable S-ovalbumin. ¹⁹ The overall change in the conformation due to the conversion is small, but the conformational change does lead to a decreased solvent accessibility of the hydrophobic core. ⁵² Accordingly, a larger negative hydration contribution likely yields the observed lower compressibility.

Attenuation increases at both non-neutral pHs confirm that heating to 60 °C and beyond induces conformational changes in ovalbumin that dissipate acoustic energy more. An opening up of ovalbumin's structure so that enhanced viscous relaxation events occur is consistent with these attenuation measurements.

Real-Time Analysis. Ovalbumin at pH 9.0 was also subjected to a heating ramp, followed by a holding phase at 70 °C. The

same effects as at pH 7.0 were observed. The relative ultrasonic velocity decreased during holding, and the magnitude of the decrease was similar at both pH values (Figure 4). In contrast, no change in the ultrasonic velocity of acidified ovalbumin held at 70 °C was apparent. The time exposed to elevated temperatures as the sample was initially heated up to 70 °C likely completed the conformational changes in the acidinduced thermally sensitive ovalbumin.

CONCLUSIONS

High-precision ultrasound measurements provide novel insights into amyloid-like protein aggregation although the role of water at the protein's surface complicates data interpretation. Changes in both the ultrasonic velocity and attenuation upon heating point to a "loosening" of molecular structure that is underway at 58 °C and is accelerated at approximately 73 °C. Fluorescence changes support such temperature-dependent transformations in the ovalbumin structure, but changes in the molecular size (from DLS measurements) that occur upon prolonged heating, especially in saline solutions, do not relate to acoustic measurements. Charge density is an important factor in inducing the growth of aggregates. Acid-induced structural changes in ovalbumin were also evident from the ultrasonic signatures. Overall, these data suggest that the amyloid-like ovalbumin aggregates consist of a compact core surrounded by loosely packed protein segments.

EXPERIMENTAL SECTION

Materials. Ovalbumin with a purity of at least 90% was obtained from Sigma-Aldrich (A5378; St. Louis, MO). All other chemicals, solvents, and reagents were also purchased from Sigma-Aldrich and were at least of analytical grade.

Sample Preparation. Ovalbumin (0.8000 g) was dissolved in degassed Milli-Q water (38 mL; Merck Millipore, Ontario, Canada) with and without NaCl (0.0584 g) without agitation to prevent the incorporation of air bubbles. Subsequently, the pH was adjusted to 2.0 or 7.0 with 1.0 or 0.1 M HCl or to 9.0 with 0.1 M NaOH and the mass was adjusted to exactly 40.00 g. All samples were prepared at least in duplicate. Next, 9.0 g of each solution was transferred to a lidded test tube and the sample was heated for 0, 5, 60, and 1200 min at 60, 70, or 80 °C in a water bath. Hence, for each heating condition, two samples were obtained, originating from two or more separate sample preparations. After heating, the samples were allowed to cool to room temperature before analysis.

ThT Fluorescence. The samples (190 μ L) were transferred to a 96-well plate, and 10 μ L of a 200 μ M ThT solution was added. Fluorescence measurements were performed with a Synergy Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT). The excitation wavelength was 440 nm, and the emission was recorded at 480 nm. Each sample was analyzed twice, and the reported values are the ratio of the fluorescence readings of the heated samples to those of the unheated samples.

ANS Fluorescence. The protein surface hydrophobicity of samples was determined with ANS as a fluorescent probe. Supernatants were diluted with 0.01 M sodium phosphate buffer (pH 7.0) to obtain a range of protein concentrations from 0.007 to 0.1 mg/mL. All diluted samples (200 μ L) were transferred to a 96-well plate, and 10 μ L of the ANS solution (8 mM in 0.01 M pH 7.0 sodium phosphate buffer) was added. The fluorescence intensity of the protein samples was measured

with a Synergy Multi-Mode Microplate Reader (BioTek Instruments, Inc.). The excitation and emission wavelengths were 390 and 480 nm, respectively. The protein surface hydrophobicity was calculated as in Chaudhuri et al. Hereto, the relative fluorescence was first calculated as the difference between the fluorescence intensities of the protein—ANS mixture and the control ANS (ANS solution with buffer without protein) divided by the fluorescence of the control ANS. The initial slope of the plot of the relative fluorescence intensity as a function of the protein concentration represents the protein surface hydrophobicity.

DLS. The samples without salt were diluted (1:1) with 50 mM NaCl before analysis, whereas the samples with salt were diluted (1:1) with a 25 mM NaCl solution. The final salt (25 mM) and protein concentrations (1.0% w/w) of all samples were thus the same. The diluted samples were examined by a Zetasizer Nano S DLS system (Malvern Instruments, Malvern, U.K.). This system employs a 633 nm laser and a fixed scattering angle (173°). The software supplied with the instrument calculates the Stokes radius from the translational diffusion coefficient. It is important to point out that the size obtained with this technique is that of a sphere moving in the same manner as the scattering protein aggregate (which is not necessarily spherical). Each sample was analyzed five times.

Ultrasonic Measurements. The ultrasonic measurements were performed with a ResoScan device (TF Instruments GmbH, Heidelberg, Germany). The ResoScan system measures the velocity and attenuation of ultrasound propagating through the solution by a resonator method.⁵⁴ The system, with two sample cells, is equipped with a Peltier thermostat to maintain a constant temperature (±0.001 °C) and can heat or cool the samples in the cells at a rate of 0.1-0.35 °C/min. Two different setups were applied. In the standard setup, the samples were heated in test tubes followed by cooling (as described above) and the ultrasonic properties were measured at 25 °C. The unheated samples were recorded as a reference. Each sample was measured at least six times. As each sample was prepared twice (see above), this corresponds to at least 12 measurements for each reported condition (e.g., pH 7.0, heated at 80 °C). In the ramp setup, the unheated samples (200 μ L) were loaded in both sample cells. The samples were heated at 0.3 °C/min from 30 °C up to either 60, 70, or 80 °C, followed by a holding phase at the final temperature. During the entire heating procedure, both the ultrasonic velocity and the attenuation were recorded. The ramp setup was performed three times for

Statistical Analysis and Data Presentation. Statistical analyses were conducted with JMP Pro software (SAS Institute, Cary, NC). Significant differences (p < 0.05) for several variables were determined using a one-way analysis of variance test. Each sample was prepared twice and analyzed multiple times depending on the analytical technique. The reported values are the averages obtained by calculating the average of the multiple measurements for each sample first and then the average of both values. The range cited is the difference between the averages of both sample formulations.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ANS, 1-anilino-8-naphthalene sulfonate; DLS, dynamic light scattering; ThT, thioflavin T

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