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The presence of heat-stable conformers of ovalbumin affects properties of thermally formed aggregates

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The aim of this work was to study the effect of the formation of more heat-stable conformers of chicken egg ovalbumin during incubation at basic pH (9.9) and elevated temperature (55°C) on the protein aggregation properties at neutral pH. Native ovalbumin (N-OVA) is converted on the hours time-scale into more heat-stable forms denoted I- (intermediate) and S-OVA, that have denaturation temperatures 4.8 and 8.4°C, respectively, higher than that of N-OVA. The conversions most likely proceed via I-OVA, but direct conversion of N-OVA into S-OVA with slower kinetics can not be excluded. It is demonstrated that both I- and S-OVA have similar denaturation characteristics to N-OVA, except that higher temperatures are required for denaturation. The presence of even small contributions of I-OVA does, however, reduce the Stokes radius of the aggregates formed upon heat treatment of the material at 90°C about 2-fold. This affects the gel network formation considerably. Since many (commercial) preparations of ovalbumin contain varying contributions of the more heat-stable forms mentioned, proper characterization or standardization of the isolation procedure of the material is essential to control or predict the industrial application of this protein.

Keywords: aggregation/ovalbumin/protein denaturation/Stokes radius/turbidity

Introduction

Control over structural stability is vital for proteins to fulfil their biological function. For example, it protects proteins against undesirable proteolytic digestion or controls desired degradation when served as a supplier of free amino acids. Structural stability is also of importance in preventing kinetic trapping of the protein in non-native conformations during its folding, which may lead to aggregation and related adverse effects. Also, (local) stability is a key element for the interaction with other biopolymers, e.g. in enzyme action. After synthesis, there are a number of ways in which proteins can use post-translational processing to fine-tune their stability and other functional properties. Chicken egg white ovalbumin is an example of a protein that in nature is found to be *N*-glycosylated on asparagine-293 (Glabe *et al*., 1980; Rago *et al*., 1992), N-terminally acetylated (Narita and Ishii, 1962) and potentially phosphorylated at serine-68 and/or -344 (Nisbet *et al*., 1981) with a dominant diphosphorylated form (Cann, 1949). Ovalbumin is the major egg-white protein (60–65%) with a molecular mass of 42.7 kDa. The protein is a non-inhibitory member of the serine protease inhibitor (serpin) super-family and its crystal structure at 1.95 Å resolution has been resolved (Stein *et al*., 1991). It has an ellipsoidal shape (70×45×50 Å) and almost the entire polypeptide chain is involved in defined secondary structure elements (α-helix and β-sheet). The protein has one solvent-accessible disulfide bridge and four free sulfhydryl groups buried in the interior of the protein.

For this protein, another process affecting the structural stability has been reported. During prolonged storage of shelled eggs, ovalbumin is transformed into a more heat-stable form, named S-ovalbumin (Smith and Back, 1965). This increased thermostability is considered to result in deterioration of the food functionality of egg white; over wide ranges of pH and temperature conditions, gels formed by S-ovalbumin forms have a lower hardness than those of native ovalbumin (Shitamor *et al*., 1984). As observed by scanning electron micrographs, the gel networks formed by S-ovalbumin also show less fine structure than native ovalbumin (Shitamor *et al*., 1984). The nature of the conversion into the S-form is still unclear. No significant difference has been detected in the primary structure, UV absorption spectra, thiol reactivity and serological properties (Smith and Back, 1965). In a number of reports the presence of an intermediate form was also mentioned with a denaturation temperature between those of the native protein and the S-form (Donovan and Mapes, 1976; Hegg *et al*., 1979). Since the formation of these heat-stable forms of ovalbumin in eggs occurs within a few days at ambient temperature, many commercial egg-white isolates contain varying but significant amounts of these more heat-stable forms of ovalbumin. This has been a continuous source of uncertainty in the interpretation of data relating to protein aggregation and network formation (see, for example, Weijers *et al*., 2002). Questions about the potential presence of more heat-stable forms of ovalbumin and the doubts regarding their participation in aggregate formation have frequently been raised. The aim of this work was to elucidate the effect of the presence of more heat-stable forms of ovalbumin in the heat-induced aggregation processes. To this end, a series of ovalbumins were prepared that were processed to different extents to obtain an increasing fraction of more heat-stable forms of ovalbumin. Of this series the aggregation kinetics at elevated temperature were studied and the aggregates formed were characterized.

Materials and methods

Preparation of ovalbumin

A batch of ovalbumin was purified from fresh hen eggs using the following semi-large-scale procedure, based on literature-described purification protocols (Vachier *et al*., 1995;

Takahashi *et al*., 1996). Egg white was separated from egg yolk by hand using nine day-fresh hen eggs (i.e. less than 6–8 h old). To the total egg white fraction (about 300 ml), 600 ml of a 50 mM Tris–HCl buffer, pH 7.5, containing 10 mM β-mercaptoethanol was added. This solution was stirred for 6 h at 4°C. Subsequently, the solution was centrifuged for 30 min at 18 000 *g* and 4°C. The pellet was discarded and 1800 ml of 50 mM Tris–HCl, pH 7.5, were added carefully to the supernatant. To the diluted supernatant, 500 g of DEAE Sepharose Cl-6B (Pharmacia) were added, followed by overnight incubation at 4°C with gentle stirring. Next, the DEAE was collected on a glass filter (G2), followed by extensive washing with 2.5 l of demineralized water and 2.5 l of 0.1 M NaCl successively. The protein was eluted stepwise with six subsequent volumes of 300 ml containing 0.15 M NaCl. The protein solution was concentrated using a Millipore ultrafiltration unit with a 30 kDa molecular mass cut-off membrane. The concentrated solution was dialyzed extensively against demineralized water at 4°C and then freeze-dried. The freeze-dried ovalbumin was stored at –40°C until further use. Generally, the yield of this procedure is about 1.1 g of ovalbumin per egg and the efficiency of isolation is about 60%. A protein purity of >98% was estimated from densitometric analysis from an SDS–PAGE gel. Electrospray mass spectrometry (results not shown) showed that the protein isolated here contained a sugar moiety of 1565 Da (linked to Asn293) with one 2-acetamido-2-deoxy-D-glucose and no mannose units as additional substituents to the common backbone (Rago *et al*., 1992). No heterogeneity in natural glycosylation could be detected.

Preparation of heat-stable forms

Heat-stable forms of ovalbumin were prepared from the above-derived ovalbumin according to the method described by Smith and Back (1965). An amount of 2.0 g of ovalbumin was dissolved in 50 ml of demineralized water and the pH of this solution was adjusted to 9.9 by dropwise addition of 0.5 M NaOH. The resulting solution was incubated at 55°C for various times ranging from 4 to 72 h. Next, the solution was neutralized to pH 7.0 with 0.5 M HCl and dialyzed extensively against demineralized water at 4°C. After dialysis, the protein was freeze-dried and stored at –20°C until further use.

Sample preparation

Ovalbumin samples were dissolved in 20 mM phosphate buffer, pH 7.0, containing 0.1 M sodium chloride and centrifuged in an Eppendorf centrifuge (14 000 r.p.m.) to remove undissolved particles (if any). Protein concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of $E_{1\ cm}^{1\%} = 6.99$. The samples were then diluted using the same buffer to a final protein concentration of 24 mg/ml used in all experiments described here, if not stated otherwise.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed on a MicroCalorimeter VP-DSC instrument (MicroCal, France). Typically, 2 mg/ml of native and (processed) ovalbumin in 20 mM phosphate buffer, pH 7.0 (degassed prior to use) were heated from 25 to 110°C at 1 K/min. The denaturation temperature (T_d) was determined using Microcal Origin 5.0 software. The total enthalpy change and the enthalpy changes of the individual components were determined by integration of the peak area upon deconvolution of the recorded thermogram by three components.

Capillary electrophoresis

The protein samples were separated on a 54 cm×50 μm i.d. polar bonded phase CElect-P150 column (Supelco, Bellefonte, PA) using a P/ACE 5500 system (Beckman Instruments, Fullerton, CA). A separation buffer of 6 M urea, 0.38 M citric acid and 0.05% methylhydroxyethylcellulose (Tylose, Hoechst, Frankfurt, Germany) with a pH of 2.5–2.6 was used. Samples (5 mg/ml) were dissolved in 6 M urea, 5 mM trisodium citrate, pH 8.0, and injected under pressure for 10 s. Migrations were run at 45°C and the voltage across the capillary was maintained at 25 kV (45 mA). A diode-array detector was used at 214 nm for detection.

Turbidity studies

The increase in the absorbance at 400 nm of a 1 ml protein sample (24 mg/ml) in a cell with a pathlength of 1 cm was monitored over time on a Hitachi U-3000 spectrophotometer at temperatures ranging from 74 to 91°C. The temperature of the cell holder could be controlled with an accuracy of ±0.1°C. The samples were gently stirred continuously using a small magnetic stirrer device. Upon injection of the sample into the cell, the temperature of the protein sample was at the desired temperature within 60 s. The time required to reach equilibration was omitted from the data analysis. The aggregation curves were analyzed by fitting of the data by the exponential function $A(t) = A_{inf}\,(1 - e^{-kt})$, where $A(t)$ and A_{inf} represent the absorption at 400 nm at time t and extrapolated to time infinity, respectively. The reaction rate k is considered to reflect the kinetics of aggregation (Li *et al*., 2001). For all samples studied the fit parameter A_{inf} appeared to be close to 3.9 (±0.2). The experiments were performed at least in duplicate.

Gel formation measurements

Gel formation of ovalbumin solutions was followed by dynamic measurements using a Bohlin CVO rheometer with a smooth concentric cylinder with C14 geometry. Measurements were performed with a target strain of 0.001 at an angular frequency of 1 Hz. To induce gel formation, samples were heated from 25 to 90°C at 1 K/min, equilibrated for 1 h at 90°C and subsequently cooled to 25°C at a cooling rate of 1 K/min. To prevent evaporation of the solvent, a thin layer of paraffin oil was put on top of the samples. The experiments were performed at least in duplicate.

Viscosity measurements

The specific viscosity of ovalbumin solutions (2.6 mg/ml 20 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl, filtered through a 0.45 μm filter) that had been subjected to heat treatment of 1 h at 90°C and subsequently cooled to 25 °C was measured with an Ubbelohde capillary viscometer at 25°C. The Ubbelohde viscometer was calibrated with the protein solution before heating. The relative viscosity of the samples was typically <1.5. Each sample was measured at least in triplicate.

Light scattering studies

Dynamic light scattering measurements of protein samples, identical with those used for the viscosity measurements, were performed using an ALV-5000 multi-bit multi-tau correlator equipped with a Spectra-Physics solid-state laser operating at 532 nm with vertically polarized light. The range of the scattering wavevectors was $3\times10^{-3} < q < 3.5\times10^{-2}$ nm^{-1}. The scattering intensities at four different angles ranging from 85 to 136° gave comparable results and the derived radii were averaged. The temperature of the sample was thermostatically

Table I. Analysis of thermograms, shown in Figure 1, obtained by differential scanning calorimetry of the ovalbumin batches differing in incubation time at 55°C and pH 9.9 using a three-component analysis

Batch (55°C, pH 9.9)	T_{d1} (°C)	T_{d2} (°C)	T_{d3} (°C)	ΔH_1 (J/g)	ΔH_2 (J/g)	ΔH_3 (J/g)	ΔH_{total} (J/g)
Native	78.3	–	–	17.3	–	–	17.3
4 h	78.6	83.3	–	11.0	7.3	–	18.3
22 h	78.8	83.4	87.2	3.8	10.4	5.0	19.2
48 h	–	83.4	86.9	–	9.1	10.6	19.7
72 h	–	83.8	86.9	–	6.0	14.1	20.1

The accuracies of determination of the denaturation temperatures (T_d) and enthalpy change (ΔH) are ±0.3 and 5%, respectively.

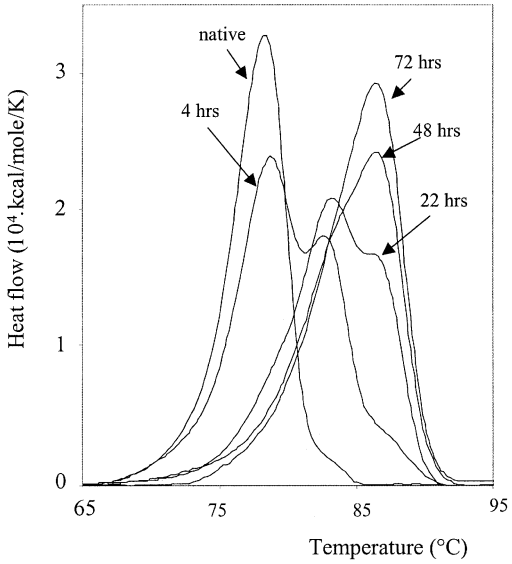

Fig. 1. Differential scanning calorimetric analysis of 2 mg/ml ovalbumin batches in 20 mM phosphate buffer (pH 7.0). The batches differ in time of incubation at pH 9.9 and 55°C: 0, 4, 22, 48 and 72 h, as indicated. The heating rate was 1 K/min in all cases.

controlled within ±0.1°C using a water-bath. The data were analyzed as described in detail elsewhere (Weijers *et al*., 2002).

Results and discussion

Formation of intermediate and S-ovalbumin

Incubation of ovalbumin in aqueous solution at basic pH (9.9) and at elevated temperature (55°C) is reported to convert the protein into a more heat-stable form. Figure 1 shows the differential scanning calorimetric thermograms of five different batches of ovalbumin that had been processed for different incubation times up to 72 h. It can be observed that the freshly prepared non-processed ovalbumin displays a symmetric band centred around 78.3°C. This material is defined here as native ovalbumin (N-OVA). After 4 h of processing at 55°C and pH 9.9, a clear additional component can be observed at 83.3°C. This component is defined here as intermediate ovalbumin (I-OVA). Upon processing for 22 h a third component at 87.2°C can be detected. This component is defined as S-ovalbumin (S-OVA). It appears that after 22 h of incubation the majority of N-OVA has been converted into either I-OVA or S-OVA. Upon prolonged incubation more I-OVA becomes converted into S-OVA. In Table I the analysis of the different thermograms displayed in Figure 1 is presented on the basis of a three-component analysis. Table II gives the distribution of

Table II. Composition of ovalbumin batches incubated for different times at 55°C and pH 9.9 based on analysis of DSC thermograms

Batch	N-OVA (%)	I-OVA (%)	S-OVA (%)
Native	100	–	–
4 h	60	40	–
22 h	20	54	26
48 h	–	46	54
72 h	–	30[a]	70[a]

The estimated error in these percentages is ~5%, except where indicated otherwise.
[a]The estimated error is ~10%.

the contribution of N-, I- and S-OVA in the various batches of ovalbumin prepared. It is clear that the formation of I-OVA from N-OVA occurs readily on the hours time-scale. Within 1 day of incubation the majority of N-OVA has been converted into I- or S-OVA. From these data it can be suggested that S-OVA is formed from I-OVA, but we cannot exclude that N-OVA is directly converted into S-OVA with slower kinetics. Even after 3 days of incubation a significant part of the protein is still present in the I-form. Attempts to prepare batches containing more than 90% S-OVA are omitted in view of the detected degradation of the protein (deamidation) that might introduce alternative differences between I- and S-OVA in functionalities. From the data presented in Tables I and II the (average) denaturation temperature (T_d) and enthalpy change (ΔH) for the N- ($T_d = 78.6 \pm 0.3$°C, $\Delta H = 740 \pm 20$ kJ/mol), I- ($T_d = 83.4 \pm 0.3$ °C, $\Delta H = 821 \pm 40$ kJ/mol) and S-OVA ($T_d = 87.0 \pm 0.2$ °C, $\Delta H = 868 \pm 45$ kJ/mol) forms can be evaluated. With increasing denaturation temperature the enthalpy change increases (by 15 kJ/K) in a way as predicted by Privalov and co-workers (e.g. Privalov and Khechinashvili, 1974). This suggests that for each of these ovalbumin forms the denaturation reflects a comparable process, i.e. (partial) protein unfolding, thereby exposing more non-polar residues to the solvent and subsequent aggregation of denatured proteins to reduce these unfavourable interactions again.

Capillary electrophoretic analysis illustrates a small but significant difference between the various batches. The migration profiles are shown in Figure 2. It can be seen that with increasing processing time a new component emerges migrating earlier from the capillary, suggesting a higher charge and/or a smaller, more compact, protein structure. The conversion of the peak eluting at 18.78 min into the peak eluting at 18.63 min follows the appearance of S-OVA rather than that of I-OVA (compare with Table II). It cannot be excluded that I-OVA elutes intermediate to these two bands.

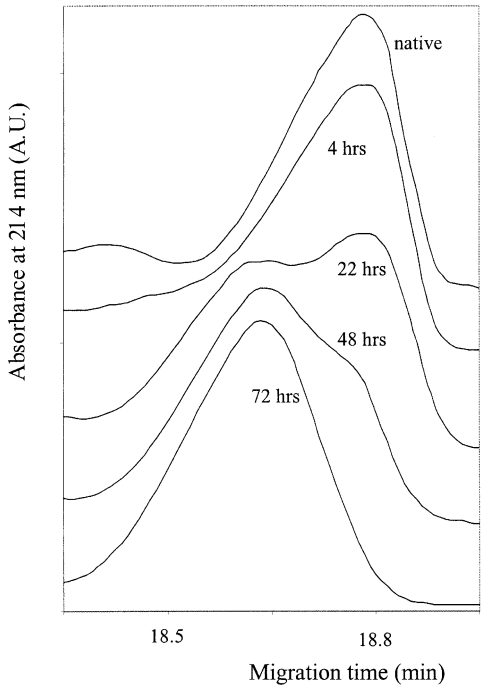

Fig. 2. Migration profiles of capillary electrophoretic analysis of five batches of processed ovalbumin. The different incubation times of the ovalbumin samples at 55°C and pH 9.9 are indicated.

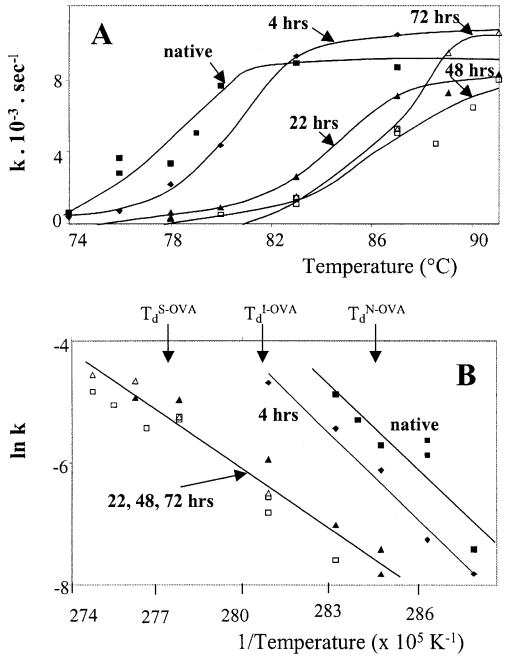

Fig. 4. (**A**) Aggregation rate of various batches of ovalbumin as determined by monitoring the turbidity at 400 nm as a function of temperature (see Figure 3), as described in more details in the Materials and methods section. (**B**) Arrhenius plot of (A). The denaturation temperatures of N-, I- and S-OVA are indicated.

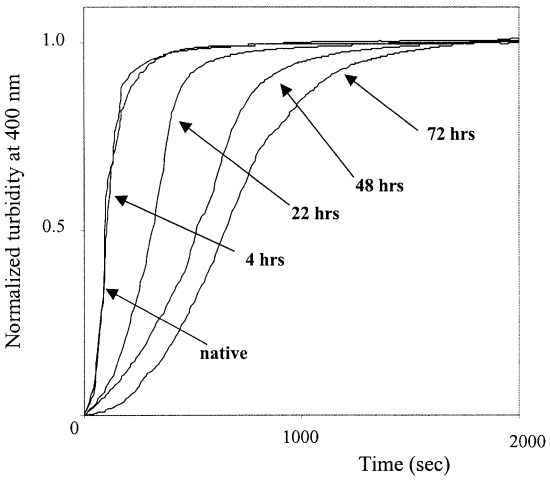

Fig. 3. Kinetics of ovalbumin aggregation as monitored by the turbidity at 400 nm in a 1 cm cuvet as a function of time of exposure of the various batches to 83.0°C. The protein concentration was 24 mg/ml in 20 mM phosphate buffer, pH 7.0, containing 0.1 M sodium chloride. The different ovalbumin batches are indicated.

Similarly to what has been reported in the literature, also between the various ovalbumin batches described here no differences could be detected using various other tools. Gel permeation chromatographic (GPC) and gel electrophoretic analysis showed that in all batches the proteins are mainly monomeric (at pH 7.0) and only a small (<5%) fraction was present as dimer. Also, tryptophan fluorescence and far-UV circular dichroism measurements could not detect any differences at a tertiary or secondary folding level (results not shown). Analytical tools to determine the number of primary amino or carboxylate groups did not show any distinction between the materials (results not shown). Determination of total sugar (mannose) content per protein gave for all batches an average of six mannose units per protein (results not shown). For the details of these determinations, we refer to Kosters *et al*. (2003).

Aggregation kinetics

Processing of ovalbumin at basic pH and elevated temperature provides materials that have a similar 'appearance', are still able to undergo a comparable thermodynamic transition, but differ significantly in denaturation temperature. Based on the disputes in the literature, the question then arises as to whether N-, I- and S-ovalbumin have a comparable follow-up process upon thermal denaturation. To test this, the kinetics of aggregation were studied by monitoring the turbidity of the batches at 400 nm with time of exposure to a defined temperature. The use of turbidity to study the kinetics of extensively aggregating proteins has been used more often (see, e.g., Lichtenbelt *et al*., 1974; Parker and Dalgleish, 1977). A typical example of such kinetics is shown in Figure 3 for the various batches incubated at 83.0°C. At this temperature it can be seen that the batch containing most N-OVA shows the fastest increase in turbidity, whereas those containing more S-OVA are much slower. By fitting the increase in the turbidity with time, the corresponding rates, reflecting the aggregation of the protein, can be obtained. These rates are presented in Figure 4A for each of the batches incubated at different temperatures. It can be observed that the batch containing N-OVA alone and that containing 40% I-OVA (processed for 4 h) are comparable whereas the three other batches overlay as well. Upon conversion of Figure 4A to an Arrhenius plot, as displayed in Figure 4B, this is even more obvious. The straight lines obtained for the native protein and the 4 h processed material are parallel, with the latter down-shifted by about

1 unit. This result can be explained since the 4 h processed sample contains 60% N-OVA (Table II) and, assuming a simple collision model, aggregation of N-OVA in this sample alone would slow the aggregation rate ~2.5-fold [i.e. $\Delta \ln k$ would differ by 1 unit as observed in Figure 4B]. Since even at temperatures close to T_d of I-OVA the aggregation does not speed up significantly, it could be suggested that I-OVA does not show any contribution to the aggregation process on the time-scales studied here and the turbidity signal is dominated by the aggregation of N-OVA.

The curves for the 22, 48 and 72 h processed batches can all be described by a single trendline. This is a remarkable result, since it covers the whole temperature range between the denaturation temperature of N-OVA and S-OVA and higher temperatures. The ~5-fold lower N-OVA concentration in the 22 h processed batch mostly explains the ~15–20 times lower aggregation rate around the T_d of N-OVA. The single straight line in the Arrhenius plot for the 22, 48 and 72 h processed samples suggests that the mechanism of aggregation appears the same for all samples (and thus for N-, I- and S-OVA), since this slope reflects the activation energy of the process.

Participation in aggregate formation

In view of the results shown in Figure 4, it can be questioned whether in the processed batches of ovalbumin described here containing different fractions of N-, I- and S-OVA, any co-aggregation of the different forms occurs. To test this, the various batches prepared by incubation for different times were heated for 1 h at a given temperature between 78 and 95°C. After centrifugation of these heated samples, the supernatant was subsequently applied to a gel filtration column at 20°C to separate aggregated (but soluble) from non-aggregated material. It was found that the fraction of non-aggregated protein coincided with the expected fraction of proteins in the batch based on the composition of forms of OVA (Table II) and their corresponding T_ds (Table I) when they would not participate in the aggregation process (results not shown). Thus, co-aggregation of the different forms of OVA requires denaturation of that OVA form. As expected, incubation at 84°C for 1 h causes all protein to aggregate (not shown).

Aggregate properties

Does the batch composition influence the properties of the aggregates formed upon heat treatment of the samples at 90°C for 1 h? According to GPC, all material is in aggregate form and the aggregates appear larger than 5×10^3 kDa (results not shown).

Remarkably, when the specific viscosity of the aggregated batches was tested it was observed that in the batch containing N-OVA only the viscosity was significantly higher compared with the batches containing also I- and S-OVA (Figure 5A). This could be indicative of larger dimensions of the aggregate composed of N-OVA alone. To verify this, dynamic light scattering analysis was performed on the derived aggregates (Figure 5B). For aggregates composed of N-OVA only, a Stokes radius of about 85 nm was found under these conditions. The presence of I- or S-OVA caused the aggregate radius to reduce about 2-fold. Assuming a common globular packing of the protein in the aggregate, one can estimate that the mass of these aggregates would be of the order of 500–600 kDa, in line with the above-mentioned particle size based on GPC. Both the viscosity and light scattering experiments demonstrated that there is no effect of the relative contribution of I- and S-OVA in the batch (Figure 5).

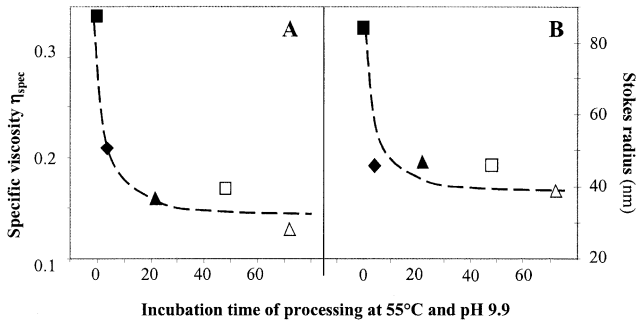

Fig. 5. Properties of aggregates formed by heat treatment for 1 h at 90°C of ovalbumin batches processed for different incubation times [native (solid squares), 4 h (solid diamonds), 22 h (solid triangles), 48 h (open squares) and 72 h (open triangles). (**A**) Specific viscosity as determined by the Ubbelohde method at 25°C for the various batches of processed ovalbumin (2.6 mg/ml) after incubation for 1 h at 90 °C. (**B**) Stokes radii of the same batches shown in (A) as determined by dynamic light scattering.

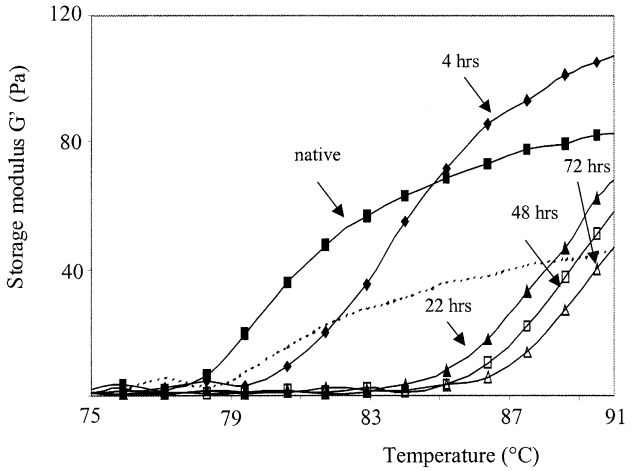

Fig. 6. Storage modulus of 24 mg/ml ovalbumin batches in 20 mM phosphate buffer, pH 7.0, containing 0.1 M sodium chloride, as determined in a rheometer. The different incubation times are indicated. The heating rate was 1 K/min.

Network properties

In view of the differences in aggregate properties modulated by the composition, it can also be expected that differences in bulk network formation might occur. This was tested by subjecting the materials to a temperature trajectory, comparable to that of the DSC experiments (Figure 1), while monitoring the development of the elastic modulus (G') of the material using a rheometer. Figure 6 shows the development of G' as a function of temperature for the various batches. It can be clearly observed that the rise in G' starts for the native material at the denaturation temperature of N-OVA. That of the 4 h processed batch is 'delayed' by about 2°C. That this is not due to the lower concentration N-OVA in the sample was demonstrated by testing a native sample at a lower concentration. This latter sample displayed a similar trace to that for the 24 mg/ml sample, but with a lower intensity (dotted line in Figure 6). This indicates that the presence of I-OVA does unambiguously affect the network-forming properties of the protein. The samples processed for 22, 48 and 72 h all show a significant G' at temperatures above 85°C, close to the denaturation temperature of S-OVA and clearly higher than

that of I-OVA. During an isothermal period at 90°C and subsequently cooling to 25°C all batches displayed an increasing G', levelling off to values between 340 and 480 Pa for all samples (not shown). Owing to the low values for the modulus, the reproducibility was not good enough in terms of absolute values to present them here.

At this point, we would like to mention that upon performing similar experiments to those described here with some commercial batches of ovalbumin, the aggregation kinetics appeared to be about 3–5 times faster. Also, upon heat treatment part of the material (10–20%) did not appear in aggregates, even upon prolonged incubation at high temperature. The size of the aggregates formed of those materials, containing generally more than 10–20% I-OVA, was also larger compared with the samples described here (results not shown). The nature of these differences is unclear, but could be related to differences in the isolation procedure. In most of the commercial preparations, ammonium precipitation steps are applied (a protocol based on Sörensen and Höyrup, 1915), which might damage the protein structure or cause co-precipitation of contaminants. Alternatively, the use of older eggs might enhance the risk of co-purification of compounds released by enzymatic processes in the egg.

Summarizing, the aim of this work was to study the effect of the formation of more heat-stable forms of ovalbumin during incubation at basic pH (9.9) and elevated temperature (55°C) on the protein aggregation properties. A single protein concentration was chosen (24 mg/ml) and used in all kinetic experiments to ensure that concentration dependences in aggregate formation and properties are not interfering with the interpretation. Moreover, since many ovalbumin preparations used do contain a variable amount of more heat-stable forms, multi-component batches were studied here, rather than that attempting to isolate individual components from these batches.

It was demonstrated in this work that by varying the incubation time, N-OVA is converted on the hours time-scale into more heat-stable forms denoted I- and S-OVA, with denaturation temperatures that are 4.8 and 8.4 °C, respectively, higher than that of N-OVA. S-OVA cannot be formed from N-OVA without the appearance of I-OVA. Whether I-OVA is a true conformational intermediate between N- and S-OVA, as suggested by capillary electrophoresis data, remains to be solved. Despite many attempts by others and in this work, the cause of the appearance of a more heat-stable form is unclear. Most likely it is related to small and local structural rearrangements, not detected by many of the biophysical or biochemical tools applied here or previously.

In many (commercial) preparations reported in the literature, there appears to be a fraction of ovalbumin that does not participate in aggregation upon heat processing. Often this is attributed to a characteristic of S-OVA. The higher temperatures required for denaturation of I- or S-OVA cannot provide an explanation for the smaller aggregates formed when I- and/or S-OVA are present. Possibly, an increased exposure of hydrophobicity, suggested to occur in I- or S-OVA (Nakamura and Ishimaru, 1981), could enforce denatured molecules to condense more rapidly into aggregates, thereby reducing the size of the aggregate particles. This would suggest that there is another mode of protein–protein interaction for the I- or S-form compared with N-OVA. The origin of these differences is a topic of current investigations. The impact of the presence of a more heat-stable form of ovalbumin on the textural properties that can be derived for this material is significant.

Overall, in this work it has been shown that deriving N-OVA as pure as possible provides the broadest potential for application of this material for desired technological purposes, since the formation of I- and S-OVA can be controlled accurately by the processing conditions. From a functional application point of view, such control allows fine-tuning of ovalbumin aggregation processes with respect to processing temperature and offers a tool to manipulate the properties of (food) matrices by controlling the type of network formed or the fraction of protein involved in the network.

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