

Effects of pH and Neutral Salts on the Formation and Quality of Thermal Aggregates of Ovalbumin. A Study on Thermal Aggregation and Denaturation

Per-Olof Hegg, Harald Martens^a and Bo Löfqvist

Biochemistry 1, Chemical Center, POB 740 S-220 07, Lund 7, Sweden, and

^a Norwegian Food Research Institute, POB 50, 1432 Aas-NLH, Norway

(Manuscript received 25 January 1979)

The effect of NaCl and CaCl₂ on the aggregation and denaturation temperature of ovalbumin was examined in the pH interval 3–11. It was found that ovalbumin had maximal thermal stability between pH values of 6–10. NaCl did not affect the denaturation temperature, while a small decrease was observed with CaCl₂ even at the low concentration range investigated. Both salts extended the pH interval of thermal aggregation, but at the alkaline side of the isoelectric point CaCl₂ suppressed the aggregation temperature more than did NaCl. The aggregation properties of S-ovalbumin were also investigated and found to be similar to those of ovalbumin, although aggregation occurred at a higher temperature level. The quality of the aggregates was described by their dry matter content. Aggregates appeared as transparent gels, opaque gels, gel-like precipitates and precipitates. The addition of NaCl gave predominantly gel aggregates at the alkaline side of the isoelectric point, while precipitates dominated when CaCl₂ was added. Gelling is an intermediate phenomenon which occurs between the two extremes of precipitating and non-aggregating conditions. Special conditions, in terms of electrostatic repulsion between the protein molecules, are required for gelling, which could be achieved by manipulating with pH, type of salt, salt concentration or added detergent. The difference between aggregation and denaturation temperature correlated with the dry matter content of the aggregates formed. When aggregation temperature was well below denaturation temperature, the resulting aggregates had a high dry matter content (precipitates). Gelling, on the other hand, was observed at an aggregation temperature equal to or above the denaturation temperature.

1. Introduction

In an earlier investigation conalbumin was used as a model protein for the examination of thermal aggregation and denaturation behaviour of food proteins.¹ It was found that changes in pH as well as additives like anionic detergents and neutral salts radically affected the thermal aggregation behaviour. The denaturation behaviour was also affected by these media variations, but in a quite different manner. The sequence of these two events appeared to be related to the quality of the thermally formed aggregates.

While accidental protein aggregation may occur during industrial processing of raw material (for example during pasteurisation or drying steps) heat setting of the texture is desired in most egg, meat and fish products. In these cases, the quality of thermally formed aggregates is as interesting as the temperature of formation.

In the present investigation we have chosen ovalbumin, the major protein in egg white, as a model protein to examine how the quality of thermally induced aggregates could be directed. The state and properties of gels are well characterised for different types of polysaccharides.² This is not the

case for proteins, even if it is known that certain proteins, e.g. ovalbumin at sufficiently high concentrations, form firm gels by heating^{3, 4} or by the addition of urea.^{5, 6} As none of the precipitates we obtained earlier with conalbumin¹ had true gel texture characteristics it was of interest to examine under what conditions thermally-formed gels could be formed. The quality of the aggregates was thus studied in different media where mainly pH, type of salt and salt concentration were varied. These variations were expected to influence mainly the charge conditions between protein molecules, which would give intermolecular interactions of different strengths. The salts selected, NaCl and CaCl₂, were used in concentrations similar to those used industrially.

In addition to aggregate quality, it was of interest to examine both the temperature of formation of the ovalbumin aggregates and the temperature of denaturation under the different conditions. Thermal aggregation⁷ processes were studied quantitatively, and separately in relation to denaturation which was measured by differential scanning calorimetry. The latter technique makes denaturation measurable in an aggregating medium. The earlier investigation of conalbumin¹ showed that coarse flocculates occurred when the aggregation temperature was registered at values below the denaturation temperature, while more viscous or milk-like precipitates were obtained when aggregation temperature was recorded above the corresponding denaturation temperature. It was postulated that the temperature of formation of the aggregates and its relation to the denaturation temperature could be used to select conditions where aggregates with particular textural character occurred.

It has been shown that egg white from eggs stored at elevated temperatures gave 'angel cakes' with reduced volume.⁸ The main change in egg white components during storage was the change of ovalbumin. The altered ovalbumin was later found to be heat-denatured at a slower rate than native ovalbumin and was named S-ovalbumin.^{4, 9-11} The transformation of ovalbumin to S-ovalbumin has been given little attention in literature until recently, when the different forms of ovalbumin were identified with differential scanning calorimetry.^{12,13} In this investigation we also compare the thermal aggregation behaviour of S-ovalbumin to ovalbumin.

2. Terminology

Aggregation is defined as any process which leads to precipitation or gelation of protein molecules into aggregates which can be visibly discerned.

3. Experimental

3.1. Materials

Ovalbumin, free from the S-variant,^{4, 9-11} was kindly prepared by Sigma Chemical Co. to our specifications (Lot No. 116 C-8220). No contamination with other egg white proteins could be detected by SDS-polyacrylamide gel electrophoresis¹⁴ or differential scanning calorimetry (DSC). A DSC thermogram of the preparation is shown in Figure 1(a) and one of an ordinary ovalbumin preparation from Sigma (Lot No. 84 C-8080) in Figure 1(b). A single peak was observed on analysis of the preparation used in this investigation while there were three peaks in the ordinary preparation corresponding to ovalbumin, intermediate ovalbumin and S-ovalbumin, respectively.¹²

S-ovalbumin was prepared by keeping a 5% (w/v) ovalbumin solution, at pH 10 and 56°C for 38 h.¹²

Sodium dodecylsulphate (SDS; Lot No. 34 C-1530) was purchased from Sigma. All other reagents were of p.a. grade obtained from Merck AG.

3.2. Methods

3.2.1. Desalting of the protein

The protein preparation was desalted on a column (100 × 2.4 cm) of Sephadex G 25 (medium). Ovalbumin (3 g) was dissolved in 150 ml of the buffer used (0.25M NH₄HCO₃) and applied to the column. After chromatography the protein was dialysed against 0.025M NH₄HCO₃ and lyophilised to constant weight. The resulting preparation contained less than 0.2% salt on a dry wt basis.

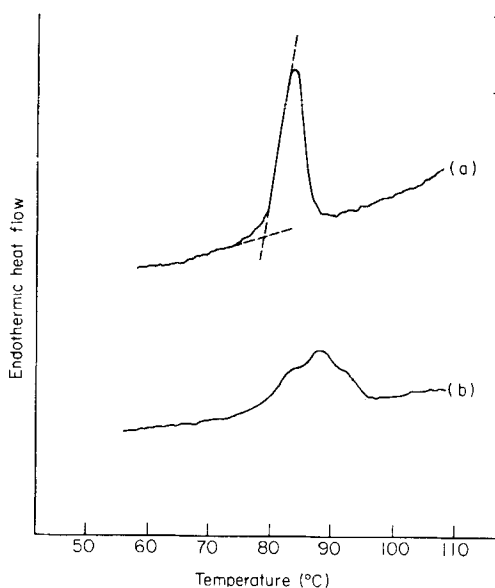


Figure 1. DSC thermograms of ovalbumin prepared from (a), fresh eggs and (b), old eggs. Both preparations were dissolved in glass distilled water at pH 8. Protein concentration was 44 mg ml^{-1} and scanning rate $10^\circ\text{C min}^{-1}$. The bar indicates an endothermic heat flow of 0.05 mcal s^{-1} . The procedure for evaluation of the denaturation temperature, T_D , is indicated in thermogram (a).

3.2.2. Preparation of ovalbumin solutions

Ovalbumin solutions were prepared as previously described,¹ except that centrifugation was applied prior to the addition of salt in order to remove insoluble material. The protein concentration was determined by $A_{1\text{cm}}^{1\%} = 7.12$.¹⁵ A concentration of 44 mg ml^{-1} was used for all experiments. The desalted protein was dissolved in deionised water and adjusted to suitable pH values with 1M HCl or NaOH (referred to as saltfree).

3.2.3. Thermal aggregation measurements

The course of thermal aggregation was registered between 25 and 95°C at a heating rate of $10 \pm 0.2^\circ\text{C min}^{-1}$. Heat treatments were carried out in glass tubes ($7 \times 75 \text{ mm}$) containing 1 ml of protein solution. The glass tubes were submerged in a beaker containing paraffin oil and an immersion heater connected to an adjustable potentiometer. The temperature was measured inside the tubes with a thermistor connected to a digital multimeter (Keithley 160). The thermistor was calibrated against a precision thermometer to an accuracy of $\pm 0.3^\circ\text{C}$. Reproducibilities and accuracies are given as three times the mean error.

The course of aggregation was monitored by removing glass tubes from the beaker at an interval of 2.5°C in the temperature region of aggregation. The tubes were immediately cooled in ice water and centrifuged at $30\,000 g$ for 45 min . The percentage of thermally aggregated protein was calculated from the decrease in absorbance of the supernatants at 280 nm . Possible effects on the u.v. absorption of the denaturation of ovalbumin were ignored. To avoid turbidity arising through dilution of the supernatants, cuvettes of a path length of 0.2 mm (Tintometer Ltd, England) were used. The percentage aggregation *vs* temperature was plotted and an aggregation temperature, T_A , was defined as the temperature at which 10% of the protein had aggregated.¹ T_A is used to describe thermal aggregation in different media and was usually determined at each 0.5 or 1.0 pH unit. The reproducibility of the T_A determination was $\pm 0.5^\circ\text{C}$.

3.2.4. Determination of dry matter content

The quality of the aggregates formed was characterised by their dry matter (DM) content. The glass tubes were weighed prior to heating (see section 3.2.3) and after centrifugation and removal of the supernatant, excess supernatant was carefully absorbed on a filter paper and the glass tubes were reweighed to give the weight of the aggregate. It should be noted that the speed and time of centrifugation was selected to avoid breakage when the aggregates appeared as gels. Assuming 1 ml of protein solution weighed 1000 mg and the concentration of soluble protein, after heating, was the same in the pellet as in the supernatant, the DM content (%) in the aggregate was calculated from

$$\frac{C_0 \times V_0 \times a}{W_a}$$

where C_0 is the initial ovalbumin concentration (mg ml^{-1}); V_0 , the initial solution volume (ml); a , percentage aggregation and W_a , the weight of the aggregate (mg).

When analysing for DM content it was observed that the aggregates ripened when the temperature was increased. This process occurred in parallel with increases in the amount of aggregated protein. Table 1 illustrates such a relationship between temperature, DM content and completeness of aggregation for S-ovalbumin in 170 mM NaCl, pH 8. It is seen that only 41% of the protein was aggregated at 85°C and that the corresponding DM content was 4.8%. At 90°C and 95°C when more than 90% of the ovalbumin had aggregated a higher and constant DM content was obtained. A similar relationship was observed at other pH values and in other media.

It was further observed that aggregation in most cases reached completeness below 95°C. The percentage DM measured at 95°C was therefore used to classify the aggregates. As aggregation in most cases was complete at this temperature, the only principal variable in the given formula is W_a , and thus the DM content can be taken as a measure of the water holding capacity of the ovalbumin aggregates. In the few cases where complete aggregation was not reached at 95°C the figure for DM content is marked with an asterisk indicating that the media conditions were close to non-aggregating conditions. The reproducibility was $\pm 3.5\%$, calculated on the measured DM contents. Complete solubility was defined as no observed phase separation after centrifugation.

Table 1. The relationship between the amount of thermal aggregation, weight of the aggregate and the DM content at different temperatures of S-ovalbumin in 170 mM NaCl, pH 8

	Temperature (°C)			
	80	85	90	95
Thermal aggregation (%) ^{a, b}	—	41.2	92.0	94.3
Weight of the aggregate (%) ^b	—	37.4	55.9	58.5
DM in the aggregate (%)	—	4.8	7.2	7.1

^a Initial protein concentration = 44 mg ml⁻¹.

^b Initial solution volume = 1 ml = 1000 mg.

3.2.5. Thermal denaturation measurements

Thermal denaturation was followed by differential scanning calorimetry, carried out on a Perkin-Elmer DSC-2 fitted with an Intracooler II cooling unit. Aluminium sample pans (Perkin-Elmer No. 219-0062) were used. A sealed sample pan which contained a volume of water equal to that of the sample (15 µlitre) was used as reference. The calibration of the DSC has been described earlier.¹ A thermal denaturation temperature, T_D , was determined at the scanning rate of 10°C min⁻¹ as shown in Figure 1(a). The peak area below T_D accounted for 5–8% of the total peak area. Thus, T_D corresponds to the onset of denaturation similarly to the determination of T_A . The reproducibility of T_D was $\pm 0.5^\circ\text{C}$.¹

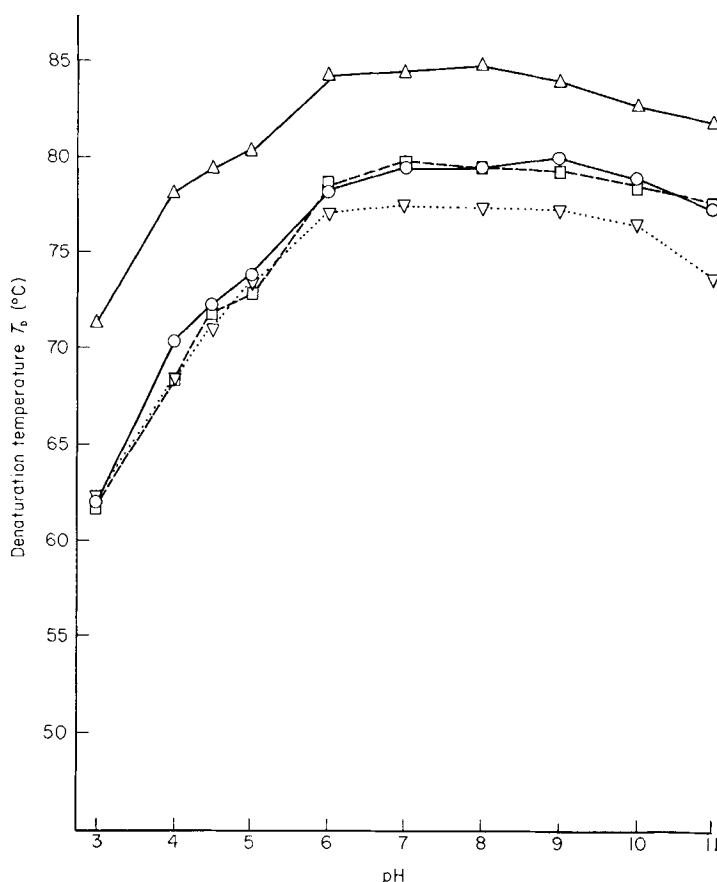


Figure 2. The denaturation temperature, T_D , of ovalbumin and S-ovalbumin as a function of pH. $\circ$, Ovalbumin in saltfree solution; $\square$, in 170 mM NaCl; and ∇ , in 17 mM CaCl₂. $\triangle$, S-ovalbumin in saltfree solution. Heating rate was 10°C min⁻¹.

4. Results and discussion

4.1. The temperature of denaturation of ovalbumin

The denaturation temperatures, T_D , of ovalbumin in various media and at different pH values are illustrated in Figure 2. In saltfree solutions T_D steadily increases from 62°C at pH 3 to around 79°C at pH 6 and then remains virtually constant until pH 10. At pH 11, however, T_D tends to decrease. Thus, the optimal thermal stability for ovalbumin molecules in saltfree solution is in the pH range 6–10.

The addition of NaCl¹ (170 mM) did not affect the thermal stability of ovalbumin (Figure 2), but CaCl₂ caused a small decrease in thermal stability corresponding to 2–3°C at all pH values above pH 5, i.e. above the isoelectric point (pH 4.5–4.9),^{16–18} even when added in such concentrations as low as 17 mM. The tendency to a lower stability at pH 11 also was more pronounced in the presence of CaCl₂ than in saltfree or NaCl solution. Concentrations of CaCl₂ in the molar range are known to destabilise protein structures,^{19,20} and our results show that such an effect can be observed already at millimolar concentrations.

When ovalbumin is transformed to S-ovalbumin T_D in saltfree solution occurred 4–9°C above those of ovalbumin (Figure 2). Such an increase in the thermostability has been described earlier at pH 8–9.¹³ According to Figure 2, however, the greatest increase in thermostability is at acidic pH values.

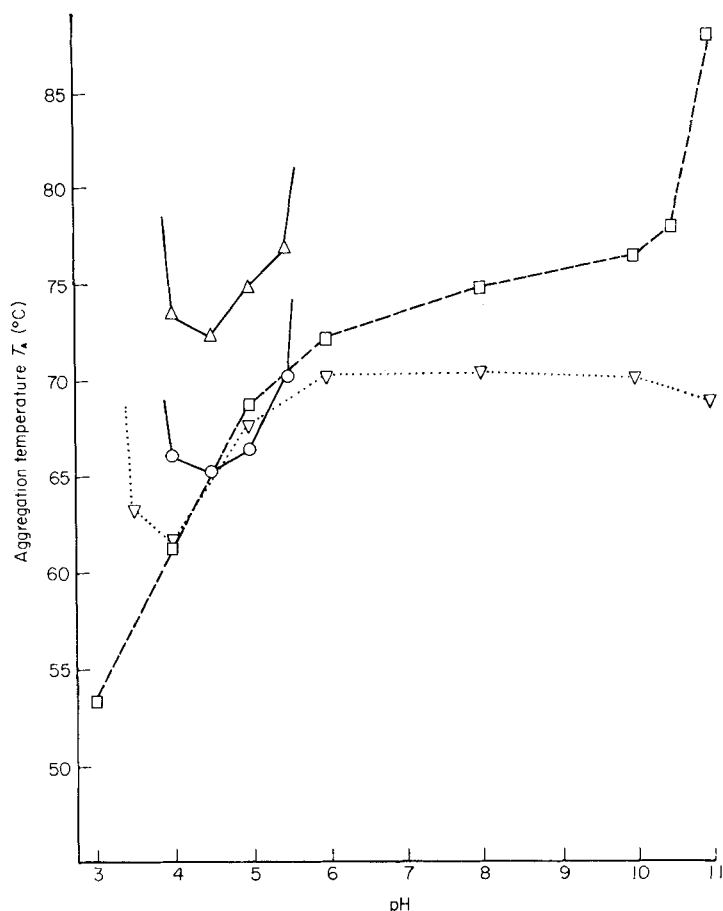


Figure 3. The aggregation temperature, T_A , of ovalbumin and S-ovalbumin as a function of pH. $\circ$, Ovalbumin in saltfree solution; $\square$, in 170 mM NaCl; and ∇ , in 17 mM CaCl₂. $\triangle$, S-ovalbumin in saltfree solution. Heating rate was 10°C min⁻¹.

4.2. The temperature of aggregate formation of ovalbumin

4.2.1. In saltfree solution

The pH-profile of the aggregation temperature of ovalbumin (T_A) in saltfree solution is given in Figure 3. Thermal aggregation occurred within the pH interval 4.0 to 5.5, i.e. a narrow interval around the isoelectric point. When the completeness of aggregation was tested by allowing the temperature to rise to 95°C, it was found that 90% or more of the ovalbumin was aggregated only between pH 4.0 and 4.75. Thus, the thermal aggregation was not complete above pH 4.75 and it diminished gradually towards higher pH-values. Similarly, there was a change towards complete solubility on the acidic side of the isoelectric point. This change was abrupt in contrast to the gradual one on the alkaline side.

A comparison of Figures 2 and 3 reveals that thermal aggregation in saltfree solution occurred 4–7°C below the denaturation temperatures. The extrapolated aggregation curve, however, crosses the denaturation curve at pH values corresponding to the end-points of the aggregation interval. Thus, thermal aggregation does not seem to be correlated to any specific change in the thermostability of ovalbumin. The fact that thermal aggregation only takes place around the isoelectric point instead indicates that the number of net charges on the ovalbumin molecule determines its aggregation behaviour.¹ The titration curve reveals that the carboxyl groups are titrated in the same region around the isoelectric point. There is thus a very steep increase in the number of

titrable groups at the acidic endpoint of the aggregation interval,¹⁶ while this increase is more gradual at the alkaline aggregation endpoint. In terms of net charge repulsion between the ovalbumin molecules this correlates well with the gradual change towards complete solubility on the alkaline side of the isoelectric point and the steep course on the acidic side.

The thermal aggregation of saltfree S-ovalbumin showed a similar pH dependency as that of ovalbumin (Figure 3). Aggregation was induced, however, about 7°C higher temperatures for S-ovalbumin. This increase in T_A is of the same magnitude as the increase in T_D (cf. Figure 2), and the resulting difference between T_A and T_D is thus virtually the same for S-ovalbumin (5–7°C) as for ovalbumin. Whether the difference between T_A and T_D indicates that the aggregation process is induced at a lower temperature than the denaturation process at equilibrium conditions¹³ or if it is a result of the heating rate selected has yet to be determined.

4.2.2. In the presence of NaCl

The pH-profile of the thermal aggregation of desalted ovalbumin was found to be very sensitive to alterations in the salt concentration. The narrow aggregation interval around the isoelectric point in saltfree solution could be extended to cover the whole range of pH values investigated (pH 3–11) by the addition of 170 mM (1%) NaCl (Figure 3). This extension may be explained by a reduction in net charge repulsion between the ovalbumin molecules caused by binding or shielding of salt ions.

A comparison of Figures 2 and 3 reveals that aggregation takes place prior to denaturation at all pH values except at pH 11. There is, however, a gradual decrease in the temperature difference $T_A - T_D$ from –6°C at pH 6 and –4°C at pH 8 to –2°C at pH 10. The aggregation curve then crosses the denaturation curve at pH 10.5, giving an aggregation temperature at pH 11 which is 10°C above the denaturation temperature. The cross point which is at pH 6 in saltfree solution thus moves to pH 10.5 on addition of 170 mM NaCl. The cross point on the acidic side of the isoelectric point which appeared at pH 4 in saltfree solution was not observed in 170 mM NaCl.

In experiments with an NaCl concentration of 85 mM (0.5%), T_A exceeded T_D at a pH between 8 and 9. The NaCl concentration was, however, still too high to give a cross point on the acidic side of the isoelectric point. The aggregation temperatures were well below T_D at all pH values, including pH 11, in the presence of 340 mM (2%) NaCl.

A comparison of the results in Figure 3 and Table 1 reveals that the difference in aggregation temperature determined for saltfree S-ovalbumin and ovalbumin is maintained also in the presence of NaCl. The T_A value was 75°C for ovalbumin at pH 8 in 170 mM NaCl and between 80 and 85°C for S-ovalbumin under the same media conditions.

4.2.3. In the presence of CaCl₂

Calcium chloride was, particularly on the alkaline side of the isoelectric point, more efficient than NaCl in promoting a thermal aggregation of ovalbumin. Thus, a concentration of 17 mM CaCl₂ caused aggregation within almost the same pH interval as 170 mM NaCl. Furthermore, the aggregation temperature was almost constant at 70°C when the solution was above pH 6, i.e. a value well below that observed with 170 mM NaCl (Figure 3). Clearly, this greater effect on T_A by calcium chloride cannot be an effect of ionic strength. It may instead be due to more specific interactions between calcium ions and the ovalbumin molecules.²¹ Such interactions on the alkaline side of the isoelectric point would result in protein molecules being more inclined to aggregate due to a decreased net repulsion. The capacity of the divalent calcium ions to form two bonds may also increase aggregation by linking different ovalbumin molecules together by their carboxyl groups.

As T_A values were measured as less than the values of T_D at all alkaline pH values (Figures 2 and 3) there was no cross point between the aggregation and denaturation curves. In fact, the temperature difference, $T_A - T_D$, was almost constant at a value of about 7°C. On the acidic side of the isoelectric point, however, 17 mM CaCl₂ did not suppress the aggregation temperature as much as did 170 mM NaCl (Figure 3). Thus, the gap between the denaturation and aggregation temperatures, results in a cross point between pH 3 and 3.5.

When the concentration of CaCl_2 was lowered, cross points were observed not only on the acidic side, but also on the alkaline side of the isoelectric point. A slight increase in the concentration of $\text{CaCl}_2 > 17$ mM however, resulted in an aggregation temperature curve which throughout the pH range investigated was in parallel to the denaturation temperature curve and thus no cross points were obtained. Since CaCl_2 already at concentrations of around 17 mM lowered the aggregation temperature more efficiently than 170 mM NaCl, we also examined if a further increase in the CaCl_2 concentration would cause a further decrease of the aggregation temperature at alkaline pH values. However, only a minor decrease was seen using 170 mM CaCl_2 except at pH 11 where a decrease of around 5°C was registered. This further strengthens the suggestion that the aggregating effect of CaCl_2 on ovalbumin is due to a specific binding of Ca^{2+} to the ovalbumin molecules.

4.3. Quality of the aggregates

As the quality of the thermally-formed aggregates is as interesting in food technology as the temperature of aggregation, we examined how quality was affected by different concentrations of NaCl and CaCl_2 in the pH range, 3–11. This was carried out by allowing the temperature to pass the aggregation and denaturation temperatures to a fixed final temperature of 95°C and measuring the DM content of the aggregates.

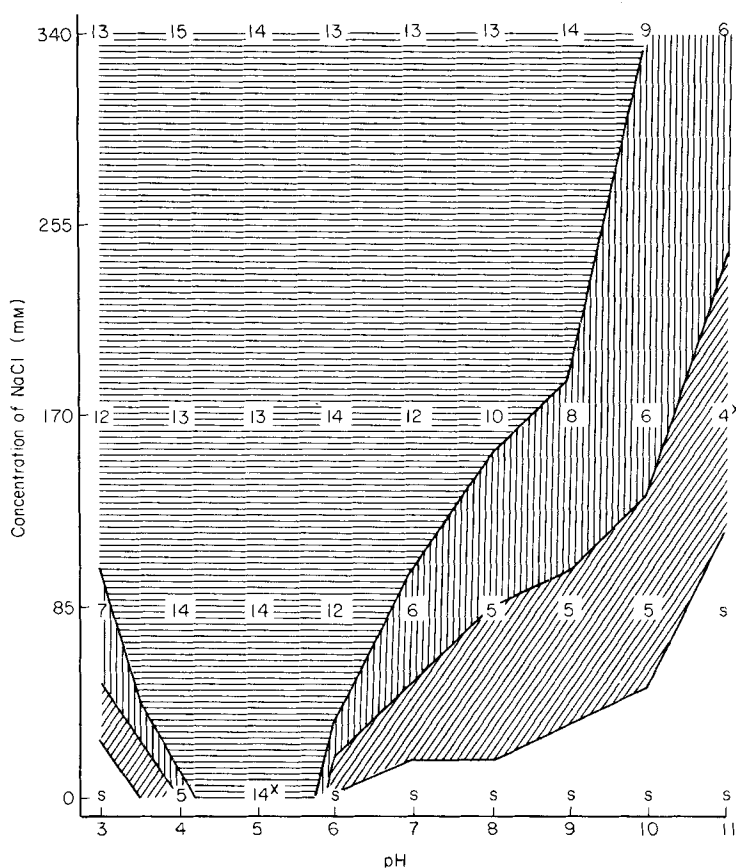


Figure 4. The DM content (%) of ovalbumin aggregates formed through heating to 95°C in different concentrations of NaCl between pH 3 and 11. Heating rate was $10^\circ\text{C min}^{-1}$. DM contents are given with figures at each pH for 0, 85, 170 and 340 mM added NaCl. s, indicates solubility. The lines differentiate areas of different DM content; ▨, below 5%; ▤, 5–9% and ▥, above 9%. The unhatched area indicates no thermal aggregation. DM contents given where aggregation was incomplete are marked with a cross.

4.3.1. *Effect of NaCl*

The DM contents of aggregates obtained at different pH values and increasing concentrations of NaCl are presented in Figure 4. The percentage of DM for aggregates formed in saltfree, 85 mM (0.5%), 170 mM (1%) and 340 mM (2%) NaCl solutions is given at each pH. Furthermore, measurements at a number of other concentrations of NaCl at various pH values enabled points of the same DM content to be connected (iso-dry matter lines). These lines were found to be suitable for the purpose of differentiating areas of different aggregate quality. Accordingly, the 5% iso-dry matter line was chosen to differentiate aggregates appearing as transparent gels (below 5%) from the opaque gels (5–9%), while these in turn were differentiated from the gel-like precipitates by the 9% iso-dry matter line.

The boundary between aggregation and solubility is also indicated in Figure 4. This phase separation line illustrates that ovalbumin will be soluble in the presence of increasing amounts of NaCl when pH is increased or decreased correspondingly from the isoelectric region. Complete solubility of ovalbumin was thus obtained at pH 6 in saltfree solution as well as at pH 11 in 0.1M (0.6%) NaCl. In fact, the line between aggregating and non-aggregating conditions has a shape similar to a titration curve for ovalbumin.¹⁶ The highest apparent net charge is thus obtained at a low concentration of salt and at pH values remote from the isoelectric point (see section 4.2.1.). By increasing the concentration of salt at such a pH the apparent net charge repulsion will decrease. NaCl could thus be looked upon as a net charge titrant and if the boundary to aggregation is supposed to be a line of equal net charge, it would have the shape of a titration curve. The observed shape of the phase separation line thus supports the suggestion that net charge repulsion is the main force that keeps the ovalbumin molecules in solution and prevents them from forming aggregates.

Similarly, the iso-dry matter lines which intersect different types of aggregates deviate more from the shape of the titration curve. This indicates that NaCl is a weak net charge titrant.

The DM content is high in the isoelectric region regardless of the salt concentration used. This is in agreement with aggregates formed at conditions of low apparent net charge. The dominating type of aggregate at the alkaline side of the isoelectric point at concentrations of up to 85 mM NaCl are transparent gels (below 5% DM content), while opaque gels (5–9% DM content) are the most frequently appearing form between 85 mM and 170 mM NaCl. At concentrations above 170 mM NaCl gel-like precipitates (9–13% DM content) occur more than transparent or opaque gels. At the acidic side of the isoelectric point the iso-dry matter lines differentiate between the same type of areas as on the alkaline side but they are narrower and occur at lower NaCl concentrations. In conclusion, Figure 4 gives a good indication of the possibilities of influencing the thermal aggregation of ovalbumin molecules into products of varying quality.

It is interesting to compare the DM content of ovalbumin (Figure 4) and S-ovalbumin (Table 1). At 170 mM NaCl, pH 8, the DM content of S-ovalbumin was 2.5% less than that of ovalbumin. Thus, the increase in denaturation temperature observed for S-ovalbumin is accompanied by the formation of aggregates with a more gel-like texture.

4.3.2. *Effect of CaCl₂*

The effect of calcium chloride on the content of DM in thermally formed ovalbumin aggregates is shown in Figure 5. The DM content found in aggregates formed on the acidic side of the isoelectric point were almost identical to those found for NaCl if compared in terms of chloride concentration (note the tenfold expansion of the concentration axis used in Figure 5). Thus, the concentration of chloride ions seems to determine the aggregation behaviour on the acidic side of the isoelectric point.

At pH values above the isoelectric point, CaCl₂ is a far more efficient precipitant than NaCl, and to be able to distinguish different quality areas it was necessary to expand the concentration axis in relation to the axis used for NaCl (Figure 4). In addition to the areas differentiated, in the presence of NaCl another iso-dry matter line at 13% could be distinguished in the presence of CaCl₂, which differentiates precipitates (above 13%) from the gel-like precipitates (9–13%). DM content as high as 19% was registered at 34 mM CaCl₂ and precipitates are the dominating form of

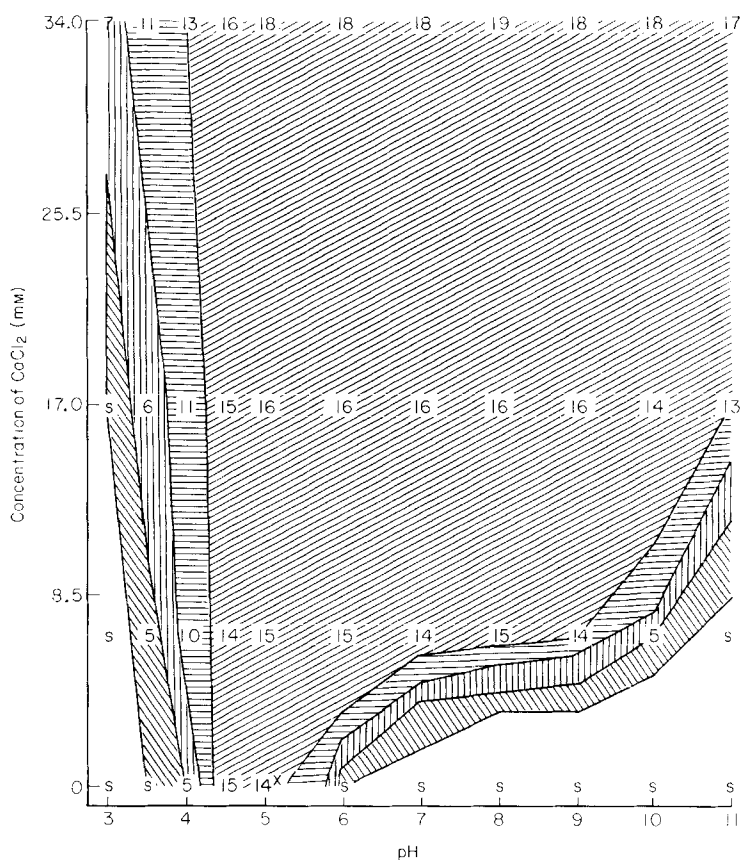


Figure 5. The DM content (%) of ovalbumin aggregates formed through heating to 95°C in different concentrations of CaCl_2 between pH 3 and 11. Heating rate was 10°C min⁻¹. DM contents are given with figures at each pH for 0, 6.8, 17.0 and 34.0 mm CaCl_2 . S indicates solubility. The lines differentiate areas of different DM content; ▨, below 5%; ▩, 5–9%; ▧, 9–13%; ▦ and above 13%. The unhatched area indicates no thermal aggregation. DM contents given where aggregation was incomplete are marked with a cross.

aggregates at all alkaline pH values while intervals dominated by gels or gel-like precipitates are very narrow. The iso-dry matter lines indicate, however, that transparent gels are formed at a concentration of CaCl_2 of approximately 3.5 mm but already at a concentration of 6–7 mm only precipitates are formed up to pH 9. If 12 mm CaCl_2 is added, transparent gels are not formed at alkaline pH values, not even at pH 11.

Consequently, the shape of the boundary between aggregating and nonaggregating conditions is, in the case of CaCl_2 , preserved also for the iso-dry matter lines and all show a profile similar to that of the titration curve for ovalbumin. From the point of net charge repulsion this indicates that calcium ions are strong net charge titrants.

4.3.3. Effect of CaCl_2 in combination with NaCl

The strong precipitating effect of calcium ions in the range pH 6–11 (Figure 5) was tested also in the presence of sodium ions. A concentration of 85 mm NaCl was chosen, since this concentration gave a broad pH-dependent variation in the quality of the ovalbumin aggregates (cf. Figure 4). Table 2 shows that the addition of even 3.4 mm CaCl_2 changed the character of the aggregates formed from gels to precipitates. The only pH where gels are now formed is at pH 10 or above. A DM content below 4.4%, as at pH 11, implies conditions of incomplete aggregation. If the concentration of

Table 2. The effect of CaCl_2 in the presence of 85 mM (0.5%) NaCl on the DM content of thermally formed ovalbumin aggregates

Concentration of CaCl_2 (mM)	DM content (%)					
	pH 6	pH 7	pH 8	pH 9	pH 10	pH 11
6.8	14.8	14.0	13.6	13.3	9.9	4.7
3.4	14.1	13.8	12.2	10.2	5.0	4.1 ^a
0	12.4	6.3	4.7	4.7	4.5	—

^a Incomplete aggregation.

CaCl_2 was increased to 6.8 mM the conditions for gel formation moved still further towards alkaline pH values, and transparent gels were formed first at pH 11, as compared to pH 8 in the absence of CaCl_2 .

The effect of CaCl_2 on the aggregation behaviour of ovalbumin in the presence of NaCl can thus be described as a suppression of the iso-dry matter lines given in Figure 4. This means, that there is an overall increase in the DM content of the aggregates and that conditions where gels and gel-like structures occur become rare. The character of NaCl as a weak aggregating agent is thus superseded by the stronger precipitating character of CaCl_2 .

4.3.4. Effect of SDS in combination with NaCl

Earlier studies on crude ovalbumin^{22, 23} as well as on conalbumin¹ showed that low concentrations of SDS could protect these proteins from thermal aggregation at neutral and alkaline pH values. It was therefore of interest to examine whether SDS had the same effect on pure ovalbumin and to what extent it would affect the character of the thermally formed aggregates. Ovalbumin dissolved in 170 mM NaCl (Figure 4) was chosen as a reference for the examination of the effect of increasing amounts of SDS. Investigations were performed at pH values ranging from 6 to 11. Lower pH values were not examined since SDS caused precipitation at pH 5 and below already at room temperature (unpublished results).

The DM content of the resulting thermal aggregates are shown in Table 3. It is seen that an increased concentration of SDS acts synergistically with an increased pH in lowering the DM content. Addition of 5 mM SDS extends the gelling area into the weak alkaline region, and at 15 mM a broad interval of transparent gels occurs between pH 7 and 10. The 17 mM-level of SDS is character-

Table 3. The effect of sodium dodecylsulphate (SDS) in the presence of 170 mM (1%) NaCl on the DM content of thermally formed ovalbumin aggregates

Concentration of SDS (mM)	DM content (%)					
	pH 6	pH 7	pH 8	pH 9	pH 10	pH 11
25	6.3	3.6 ^a	—	—	—	—
17	5.5	4.9	3.8 ^a	3.6 ^a	3.2 ^a	—
10	7.5	4.8	4.7	4.7	4.5	—
5	10.8	5.6	5.2	4.8	4.6	3.6 ^a
0	13.8	11.9	9.6	7.8	5.5	3.5 ^a

^a Incomplete aggregation.

ised by DM contents below 4.4%. As pointed out earlier, this implies incomplete aggregation and the aggregates appeared as loose or viscous gels. Apparently these gels are close to conditions where solubility occurs. This was also confirmed by increasing the concentration of SDS to 25 mM where the pH interval of gel formation decreased at the expense of nonaggregating conditions and thermal aggregation was prevented above pH 7.

4.4. Relation between the denaturation and aggregation temperatures and the quality of the aggregates

The preceding sections show that gels and gel-like precipitates appear as intermediate zones of aggregation between precipitation and complete solubility. It is also evident that the intermediate zones vary in width depending on factors such as pH, concentration of neutral salts and addition of SDS, and that in some media the zones are extremely narrow and difficult to detect.

Compared to the denaturation and aggregation temperatures given in sections 4.1. and 4.2. it is obvious that precipitates appear under media conditions where the aggregation temperature is registered well below the denaturation temperature. From the data presented on ovalbumin (Figures 2 and 3) four examples can be identified where the gap between the aggregation and denaturation temperatures decreases and the point at which the aggregation curve finally crosses the denaturation curve. The resulting four cross points are found just below pH 4 and 6 in saltfree solution, pH 10.5 in 170 mM NaCl and around pH 3.5 in 17 mM CaCl₂. These points are located in or adjacent to zones of transparent gels in Figures 4 and 5.

A more detailed comparison of the temperature difference $T_A - T_D$ and the state of aggregation reveals that precipitates (DM content above 13%) are formed at an aggregation temperature which under the applied measuring conditions is registered around 6–7°C below the denaturation temperature. Similarly, gel-like precipitates i.e. aggregates with a DM content of 9–13% appear at a temperature 4–6°C below the denaturation temperature, while opaque gels (5–9%) are found at temperature differences of around 2–4°C. Transparent gels appear around the cross point, at temperatures 1 or 2°C below the denaturation temperature, but also at temperatures well above this temperature. It is evident that large positive differences are found close to non-aggregating conditions, as T_A in these cases usually increases. Consequently, most of the conditions in Table 3 are characterised by large positive differences between T_A and T_D .

It should be pointed out, however, that T_A and T_D are strictly related to a linearly increasing temperature¹ of 10°C min⁻¹ and that the temperature difference $T_A - T_D$ would attain different values if measured at any other rate of heating.¹ In spite of this it is evident, that aggregates ending up with a high DM content are induced at an early stage of thermal denaturation and it is tempting to assume that the proceeding denaturation contributes to the formation of dense aggregates. Equally evident is the aggregates with a low DM content induced at a late stage of thermal denaturation. It therefore seems as if an initially more denatured protein structure accompanies gel formation in contrast to the situation which gives rise to precipitation. Thus, more extended molecular structures might allow the development of a three-dimensional network of protein molecules constituting the framework of a gel.

In terms of net charge repulsion gels seem to be formed between the high level which characterises solubility and the low level which will give rise to precipitation. By increasing the net charge repulsion of a medium giving gels (increased pH, decreased salt concentration, increased concentration of SDS) the gels become increasingly transparent and a repulsion strong enough to prevent intermolecular interactions is finally achieved. Gelling conditions thus correspond to a situation where the aggregating forces set free by denaturation are just strong enough to counteract the electrostatic repulsion.¹

Acknowledgements

We thank Ingun Sundén-Cullberg and Berit Overrein for technical assistance and Dr Bengt Jergil for helpful discussions. This investigation was supported by grant No. 76-3698 from the Swedish Board of Technical Development and by the Agricultural Food Research Society, Norway.

References

1. Hegg, P.-O.; Martens, H.; Löfqvist, B. *J. Sci. Food Agric.* 1978, **29**, 245.
2. Rees, D. A. *Chem. Ind. (London)* 1972, **16**, 630.
3. Holme, J. J. *Phys. Chem.* 1963, **67**, 782.
4. Smith, M. B. *Aust. J. Biol. Sci.* 1964, **17**, 261.
5. McKenzie, H. A.; Smith, M. B.; Wake, R. G. *Biochim. Biophys. Acta* 1963, **69**, 222.
6. Frensdorff, H. K.; Watson, M. T.; Kauzmann, W. J. *Am. Chem. Soc.* 1953, **75**, 5157.
7. Foster, J. F.; Rhees, R. C. *Arch. Biochem. Biophys.* 1952, **40**, 437.
8. Meehan, J. J.; Sugihara, T. F.; Kline, L. *Poultry Sci.* 1962, **41**, 892.
9. Smith, M. B.; Back, J. F. *Aust. J. Biol. Sci.* 1965, **18**, 365.
10. Smith, M. B.; Back, J. F. *Aust. J. Biol. Sci.* 1968, **21**, 539.
11. Smith, M. B.; Back, J. F. *Aust. J. Biol. Sci.* 1968, **21**, 549.
12. Donovan, J. W.; Mapes, C. J. *J. Sci. Food Agric.* 1976, **27**, 197.
13. Donovan, J. W. *J. Sci. Food Agric.* 1977, **28**, 571.
14. Neville, Jr, D. M. *J. Biol. Chem.* 1971, **246**, 6328.
15. Glazer, A. N.; McKenzie, H. A.; Wake, R. G. *Biochim. Biophys. Acta* 1963, **69**, 240.
16. Cannan, R. K.; Kibrick, A.; Palmer, A. H. *Ann. N.Y. Acad. Sci.* 1941, **41**, 243.
17. Gilbert, A. B. *Physiol. Biochem. Dom. Fowl* 1971, **3**, 1291.
18. Rhodes, M. B.; Azari, P. R.; Feecey, R. E. *J. Biol. Chem.* 1958, **230**, 399.
19. von Hippel, P. H.; Wong, K. Y. *J. Biol. Chem.* 1965, **240**, 3909.
20. von Hippel, P. H.; Schleich, T. *Structure and Stability of Biological Macromolecules*, 2 (Timasheff, S. N.; Fasman, G. D., Eds), M. Dekker, Inc, New York, 1969, p. 417.
21. Takashima, S. *J. Polymer Sci.* 1963, **1**, 2791.
22. Hegg, P.-O.; Löfqvist, B. *J. Food Sci.* 1974, **39**, 1231.
23. Hegg, P.-O.; Löfqvist, B. *J. Sci. Food Agric.* 1977, **28**, 103.