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Gelation of a Model Globular Protein

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Summary: Hen egg white lysozyme (HEWL) was exposed to various physical and chemical denaturing environments to encourage protein denaturation and consequent gelation. Its phase behavior was examined as a function of pH, temperature and also in the presence of the reductant dithiothreitol (DTT). Transparent viscoelastic gels form at low pH values while opaque gels form under alkaline conditions. No increase in viscosity was observed for systems in pure water unless 20 mM of DTT was added, which is known to break the disulfide bridges present in HEWL. The microstructure of the gel was studied using transmission electron microscopy (TEM) and environmental scanning electron microscopy (ESEM). Gels formed at low pH contain fibrils ~10 nm in diameter with various lengths while at high pH the gels are dominated by particulate aggregates. Thinner fibrils that are 4–6 nm in diameter are observed in the gels formed in the presence of DTT. In this case the distinct feature of the gels is they are thermoreversible and can be melted and reformed easily by varying the temperature.

Keywords: electron microscopy; hydrogel; microstructure; self-assembly

Introduction

Hydrogels have recently attracted much interest in the biomaterials sector because of their ability to entrap large quantities of water or biological fluids. They are traditionally fabricated with high molecular weight amphiphilic polymers cross-linked through physical entanglements or covalent bonds. Recently the ability of proteins and peptides to self-assemble into ordered supramolecular architectures on the meso- to macroscopic length scales has attracted considerable attention in the development of novel biomaterials due to their potential biocompatibility and biodegradability.[1–4] In particular, the β-sheet motif is of most interest due to its important role in the formation of protein fibrils.[5] These fibrils have recently been shown to further self-organize into a three-dimensional network, i.e. a protein hydrogel that is able to retain up to 97% water or biological fluid.[3] As with natural globular proteins, it is known that mild denaturing conditions, such as low pH, elevated temperature and organic solvents encourage the formation of fibrils rich in β-sheet structures[6] and if the protein concentration is above a critical threshold, fibrils will self-organize to form a three-dimensional fibrillar network.[4] However, systematic studies on protein self-assembly in response to different destabilizing conditions and consequent effects of such destabilizing conditions on the morphology of resulting supramolecular structures are few in the literature. In this work, we have focused on the gelation behavior of the model protein hen egg white lysozyme (HEWL) under various physical and chemical denaturing conditions. This protein is a small globular protein and contains both α-helix and β-sheet in its secondary structure and has a high solubility in water. Various conditions that can encourage fibrillation and gelation were investigated and the

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morphology of the resulting gels was subsequently explored.

Materials and Methods

Materials

HEWL was purchased from Sigma and used without further purification. The desired quantity of protein was dissolved in doubly distilled water with and without 20 mM reductant dithiothreitol (DTT) (Fluka). HCl and NaOH were used to adjust the pH to 2 and 12 respectively, when required. The protein solution was agitated for 60 seconds using a vortex mixer and the sample was used immediately for gelation studies or characterization experiments.

Transmission Electron Microscopy (TEM)

TEM experiments were performed using a CM200 TEM from Philips at 200 kV accelerating voltage. In order to investigate the fibrillar structure of our material, gels were diluted 20 fold and agitated vigorously before a 5 μL drop of the resulting solution was applied to a carbon coated Formvar grid. The grid was blotted after 60 seconds and left to air dry. A 5 μL drop of 2% (wt/vol) uranyl acetate (Agar Scientific) solution was subsequently placed on the grid, blotted after 30 seconds, air dried and examined under the TEM.

Environmental Scanning Electron Microscopy (ESEM)

Gel samples were examined by placing a drop of sample on a copper stub inside the microscope chamber of an FEI Quanta 200 ESEM. The samples were left to equilibrate at 5 °C (temperature controlled by a Peltier device under the copper stub). Subsequently a few drops of distilled and deionised water were placed around the sample, before sealing and evacuating the chamber to an initial pressure of 8 Torr. The chamber was flooded several times with water vapor before reducing the chamber pressure to ~6 Torr. Images were subsequently taken using an accelerating voltage of 10 kV.

Micro Differential Scanning Calorimetry (microDSC)

MicroDSC measurements were performed using a SETARAM micro differential scanning calorimeter III. The instrument was calibrated using the SETARAM ''Joule effect'' calibration module supplied with the instrument. The sample cell was filled with 0.5–0.6 mL protein solution using a micropipette. The reference cell was subsequently filled and its weight adjusted using a micro balance to ensure an identical mass of solution was present in both the sample and reference cells. The reference was either pure water or DTT/water mixture. MicroDSC thermographs were recorded using a 1.0 °C min^{-1} scanning rate in the temperature range 10 to 90 °C. A minimum of 3 heating and cooling cycles were performed for each sample and each measurement was repeated at least 3 times to ensure reproducibility.

Results and Discussion

Non-Reductive Conditions

In order to investigate the effect of pH on the gelation behavior of lysozyme, lysozyme solutions at 2 mM were prepared at pH 2, pH 7 and pH 12 and incubated at 65 °C for varying periods of time. We found that gel formation is largely dependent on pH. At pH 2 transparent gels were observed after 48 hours incubation at 65 °C (Figure 1A). However, at pH 7 the protein solution remained liquid-like and no increase in viscosity was observed after 14 days incubation (Figure 1B), and no increase in viscosity was observed even when the temperature was increased to 85 °C. At pH 12 opaque gels formed in 5 minutes at 65 °C (Figure 1C).

The morphology of transparent gels formed at pH 2 was firstly investigated using ESEM. However, we did not observe any microstructure in ESEM initially at 6.0 Torr which is likely to be due to the presence of a large amount of surface water. By slowly dehydrating the sample inside the ESEM chamber to remove

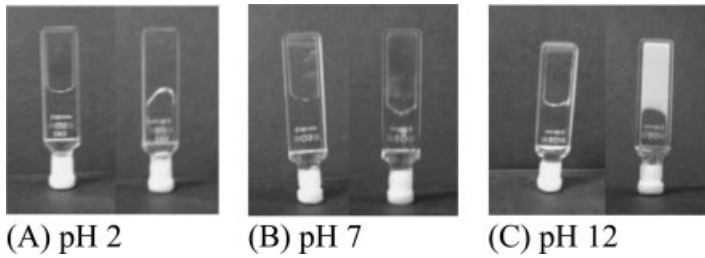

Figure 1.
Lysozyme samples dissolved in water at A) pH 2, B) pH 7 and C) pH 12 before (left) and after (right) incubation at 65 °C.

the surface water, still no structure is visible. We think this is possibly due to the dimension of fibrils and the resulting network being outside resolution of the instrument. Thus we turned to TEM which was used to monitor the morphology of the diluted gel and a typical micrograph is given in Figure 2A. The image reveals the presence of long fibrillar structures in the diluted gels. Their diameter is ~10 nm which is in the range of ~6–13 nm reported for other protein fibrils in the literature such as β-lactoglobulin fibrils.[7] The fibrils have various lengths and are usually twisted to form larger fibers as shown in Figure 2B. Similar helical arrangements have also been observed for other protein fibrils.[8]

ESEM micrographs of opaque gels formed at pH 12 are shown in Figure 3. It can be seen in this case gels are composed of amorphous particulates in contrast with the fibrillar structure observed for the transparent gels formed at pH 2. Slowly dehydrating the sample inside the ESEM chamber reveals the diameter of the particulates is ~2 μm (Figure 3B).

Here we have shown the gelation behavior of lysozyme and the structure of resulting gel networks are highly dependent on pH. This is mainly because pH affects the charge state of the protein. At pH 2 we are far from the isoelectric point of lysozyme (~11) and protein molecules are positively charged. Consequently a competition arises from the repulsive electrostatic interactions and the attractive interactions that arise from the exposed hydrophobic patches in the partially folded

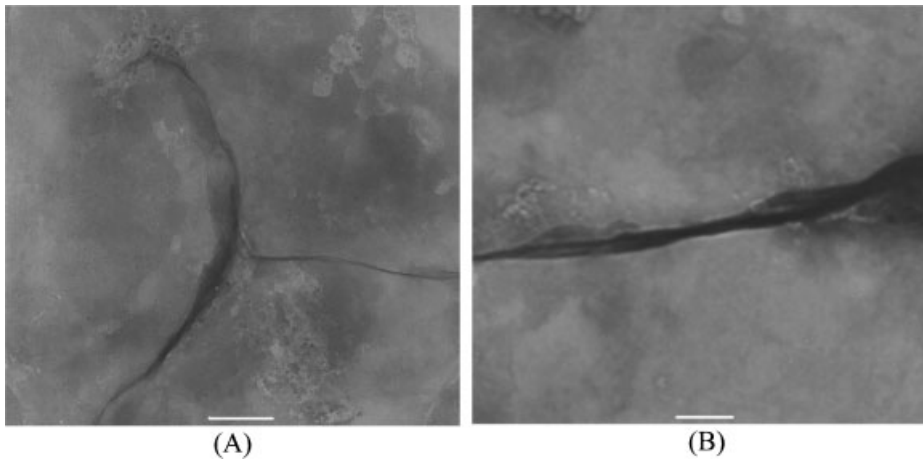

Figure 2.
TEM micrographs of negatively stained fibrils from a 20-fold diluted lysozyme gel formed at pH 2 where the scale bar represents A) 200 nm and B) 100 nm.

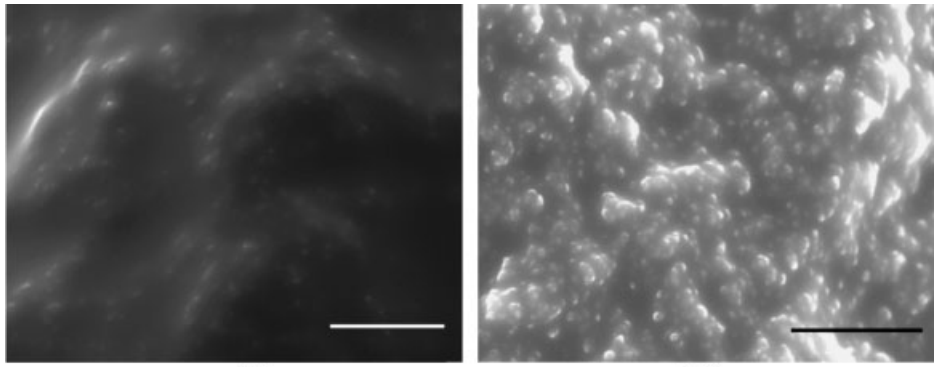

Figure 3.
ESEM micrographs of the lysozyme gel formed at pH 12 with the chamber pressure at (A) 6.00 Torr, (B) 5.50 Torr, scale bar = 50 μm.

state. As a result, the formation of aggregate nuclei is substantially limited; therefore the protein can undergo substantial conformational reorganization in response to high temperatures. In contrast at pH 12 the net charge of the protein is low, thus the aggregation process occurs immediately when the temperature is increased and the resulting gels are dominated by amorphous spherical particulates. At neutral pH the protein is positively charged, however, the denaturing conditions are mild where the exposed hydrophobic patches are not sufficient for the protein to self-associate, thus no evident aggregation is observed and the sample remains transparent and liquid-like.

Reductive Conditions

As shown above no gel forms at pH 7 which is the physiological condition required for any biological application. Thus the reductant DTT was added to the system in order to disrupt the disulfide bridges in the protein and provide the protein backbone with more flexibility which could encourage protein unfolding and subsequent gelation. We found by doing this we could obtain a clear gel in the presence of 20 mM DTT upon heating the lysozyme solution to 85 °C, incubating for 10 minutes and cooling slowly to room temperature. The gel is strong and self-supporting at the lysozyme concentration of 3 mM or above (Figure 4).

The morphology of the diluted gel was examined using TEM and a typical micrograph is given in Figure 5A. Thin fibrils which are ~4–6 nm in diameter are observed in the diluted gels. This suggests that the disruption of the disulfide bonds, which is known to stabilize the partially folded state, does indeed facilitate the fibrillation of lysozyme. The fibrils in TEM appear short and stiff and would not be expected to entangle and form a three-dimensional network. However, it is likely as suggested by Figure 5B that these fibrils align along their long axes to form larger fibrillar structures.

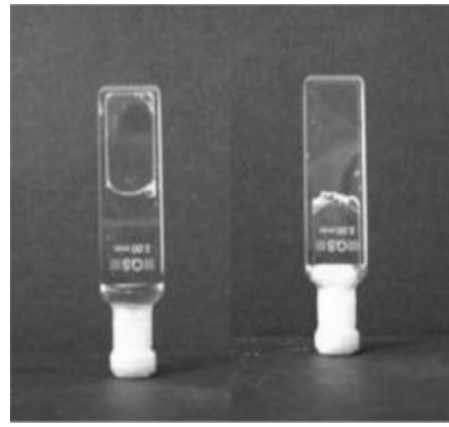

Figure 4.
Lysozyme solution (3 mM) in a DTT/water mixture before (left) and after (right) a heating/cooling cycle (25–85 °C).

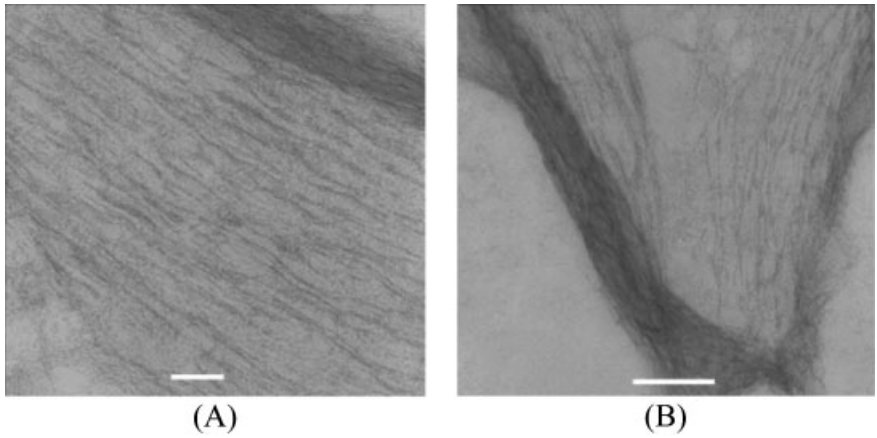

Figure 5.
TEM micrographs of negatively stained fibrils from a 20-fold diluted lysozyme hydrogel in the presence of DTT where the scale bar represents (A) 100 nm and (B) 200 nm.

The difference in the thermal behavior of lysozyme with and without DTT was explored further using microDSC. For the protein sample in pure water a single endothermic peak is observed at $75.8 \pm 0.5\,^{\circ}C$ (Figure 6A) during the first heating run which is associated with the denaturation of native lysozyme.[9] Upon cooling an exothermic transition is observed at the same temperature which

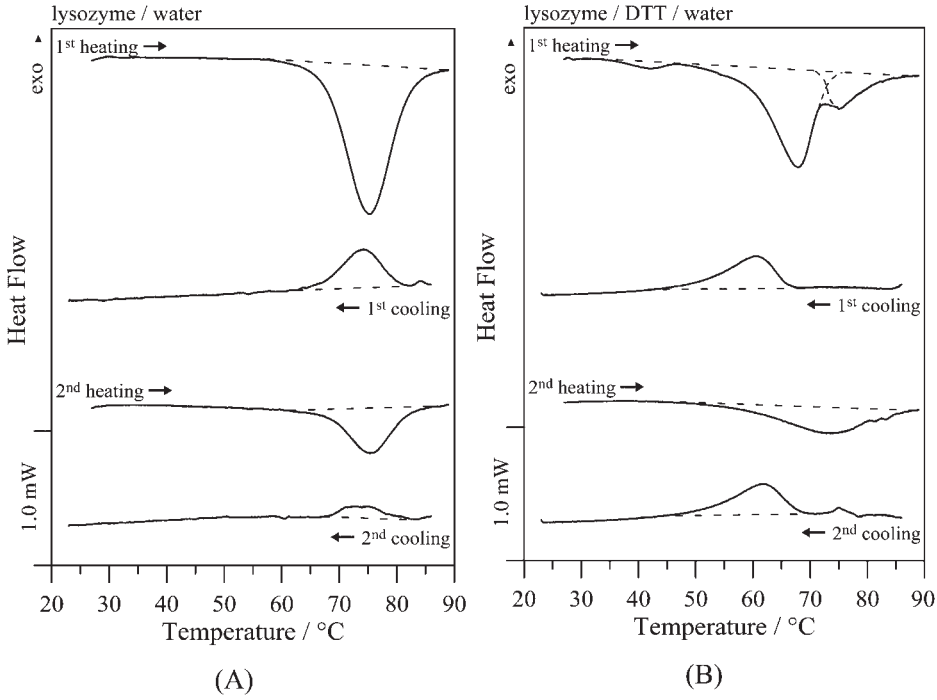

Figure 6.
MicroDSC thermographs of lysozyme samples (3 mM) in (A) pure water and (B) in a DTT/water mixture during two heating/cooling cycles.

is due to the partial renaturation of the protein. During the second thermal cycle the denaturation and renaturation transitions of lysozyme are again observed at the same temperature and the associated enthalpies decrease in comparison with the first thermal cycle. Such behavior suggests that during each heating cycle a portion of the lysozyme is denatured irreversibly and as the number of thermal cycles increases, more lysozyme denatures irreversibly. It should be emphasized that no gelation is observed for lysozyme at pH 7 without DTT.

For the solution prepared in a DDT/water mixture three endothermic transitions are observed during the first heating at 41.7 ± 0.5, 67.8 ± 0.5 and $74.5 \pm 0.5\,^{\circ}C$ (Figure 6B). The first endothermic transition detected at $41.7 \pm 0.5\,^{\circ}C$ is thought to be due to the partial unfolding of lysozyme because of the lower stability of lysozyme in the presence of DTT. The second transition observed at $67.8 \pm 0.5\,^{\circ}C$ is associated with protein denaturation which is reproducibly $\sim 8\,^{\circ}C$ degrees lower in comparison to the lysozyme solution prepared without reductant. The third endothermic transition during the 1st heating cycle is observed at $74.5 \pm 0.5\,^{\circ}C$. Since its temperature is in the melting range of lysozyme gels it is likely that the origin of the peak is the melting of aggregates or links that have formed during the first heating cycle of the sample. Supporting evidence comes from infrared spectra of the lysozyme sample with DTT in a heating/cooling cycle (results not shown). During the subsequent cooling of the lysozyme in DTT/water mixture a single exothermic transition at $60.3 \pm 0.5\,^{\circ}C$ is observed (Figure 6B). This transition temperature correlates well with the macroscopic gelation temperature observed by visual inspection of the sample upon cooling and suggests that the transition corresponds to the gelation of the lysozyme/DTT/water system. On the second heating the microDSC thermograph features a single broad endothermic transition centered at $73.4 \pm 0.5\,^{\circ}C$. This corresponds to the macroscopic melting of the gel and the transition temperature is in good agreement with the macroscopic melting temperature observed visually during heating. Figure 6B shows that the same gelation peak is again observed during the second cooling, indicating that the gel formation is thermoreversible. It should be noted that in further heating/cooling cycles, almost identical gelation and melting transitions are observed, thus confirming that lysozyme gelation in the presence of DTT is indeed thermoreversible.

Conclusions

The gelation of lysozyme has been shown to be dependent on both its physical and chemical environments. Without the presence of the reductant, two types of the gel have been obtained: transparent fibrillar gels which form at elevated temperature and low pH and opaque gels which form at elevated temperature and high pH and are composed of amorpous particulates. The addition of the reductant DTT destabilises the native structure of the protein, thus encouraging its partial unfolding under mild denatruing conditions. This leads to the formation of a thermoreversible fibrillar hydrogel at physiological pH.

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