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Maillard induced complexes of bovine serum albumin — a dilute solution study

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Abstract

Association of bovine serum albumin (BSA) on heating in the presence and absence of 2% xylose has been studied using dynamic light scattering and sedimentation velocity. When 3% solutions of the protein alone are heated at 95°C association products are formed with molar masses of $\sim 2 \times 10^6$ g/mol, a value which is independent of the time of heating. These aggregates can be dissociated in solvents that disrupt non-covalent bonds. When the reducing sugar xylose is present there is a continuous change in the hydrodynamic properties with time. After 80 min a molar mass in excess of 7×10^6 g/mol is obtained. This increase in molar mass is attributed to additional non-disulphide linkages resulting from the Maillard reaction. Information about the gross conformation of the Maillard induced association products has been obtained from MHKS (Mark-Houwink-Kuhn-Sakarada) double logarithmic plots of $D_{20,w}$ and $s_{20,w}$ against molar mass. The values of the MHKS coefficients obtained are most consistent with a linear rod: i.e. the association is of an end-to-end type.

Keywords: Maillard reaction; Sedimentation coefficient; Diffusion coefficient

1. Introduction

It is recognised that protein crosslinks can be affected by the Maillard reaction. This can have an important effect on protein nutritional quality [1] and on the structure of tissue during the ageing process [2].

One particular interest in Maillard crosslinking of proteins is as a means of enhancing thermal gelation. If globular proteins are heated in the presence of a reactive reducing sugar such as xylose the threshold concentration required for gelation is lowered [3]. This is partly a consequence of a fall in pH occurring at high temperatures as a result of acidic byproducts of the Maillard reaction. However, it is also clear that additional covalent crosslinks have been formed since in contrast to conventional gels the Maillard gels were insoluble in solutions of 1% sodium dodecyl sulphate plus 1% β-mercaptoethanol. Most of these studies have been carried out on gel samples produced by heating solutions of BSA for 121°C for 30 min, conditions which are relevant to food sterilization processes.

The objective of the work described in this current paper was to detect the earlier stages of the crosslinking process which we postulated should occur when BSA/xylose mixtures were heated under less severe conditions. By using a combination of sedimentation velocity and dynamic light scattering we have obtained information about the molar mass and the gross conformation of the association products formed.

2. Experimental

2.1. Materials

Bovine Serum Albumin (BSA) and D(+)xylose were obtained from Sigma Chemical Co. Ltd.

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2.2. Sample preparation

Solutions of 3% (w/v) BSA and 3% BSA + 2% xylose were prepared by dispersing the components in deionised distilled water with stirring. Ten-millilitre aliquots of these solutions were placed in 25-ml glass bottles, sealed and incubated in a water bath at 95°C for a range of times: between 10 and 80 min (at longer times the BSA/xylose samples began to gel). Samples were allowed to cool and then, after pH measurements had been recorded, used in the original or diluted form to carry out subsequent studies.

BSA solutions (3% (w/v)) were also prepared using deionised distilled water that had been passed through a 0.2 μm Millipore filter. The pH of this solution was then lowered using 1 N HCl to a value of 6.30 and heated at 95°C as previously described. Filtered water was used since the final samples would not pass through a 0.45 μm filter due to the formation of large aggregates, the procedure usually used to clarify the samples prior to obtaining diffusion coefficient measurements.

Samples of the low pH BSA and BSA/xylose solutions were also prepared for analysis by dynamic light scattering after heating at 95°C for 20 min. Solvents used to dilute these samples to the required 3 mg/ml for the experiment included 1% sodium dodecyl sulphate (SDS), 1% SDS + 1% β-mercaptoethanol (β-ME) and phosphate chloride buffer (0.1 M) at pH 6.8. These were all carefully prepared using filtered deionised distilled water.

2.3. Diffusion coefficient measurement: dynamic light scattering

A Malvern Instruments (Malvern, UK) System 4700C dynamic light scattering photometer was used to determine the translational diffusion coefficient for the BSA and BSA/xylose samples. This system consisted of a 40 mW He-Ne laser, a water bath maintained at 25.00 ± 0.05°C, a filter and pump system for cleaning the water bath, a variable angle photomultiplier and a 64-channel correlator. To process the data and obtain diffusion coefficients an I.P.C. personal computer using Malvern Automeasure software was employed.

To clarify the solutions and remove dust, the samples were passed through a 0.45 μm Millipore filter and into the light scattering cells using a sterile hypodermic needle. The cells were kept dust-free according to a procedure similar to that described by Sanders and Connell [4].

In order to obtain a mean (z-average) diffusion coefficient, values were calculated from five measurements with an experimental time of 120–1000 s and a sample time of 2–15 μs. The scattered light was measured at an angle of 90° to that of incident light.

The diffusion coefficient values obtained were converted to standard conditions (a temperature of 20°C and the viscosity of water as a solvent at 20°C) using the following equation [5];

$$D_{20,w} = \frac{293.1\ \eta_{T,b}}{T\ \eta_{20,w}} D_{T,b} \quad (1)$$

where $\eta_{T,b}$ is the solvent viscosity and $\eta_{20,w}$ is the viscosity of water at 20°C.

For the values obtained for the samples diluted with solvents other than water ratios of $\eta_{T,b}/\eta_{20,w}$ had to be determined. This was carried out using an Ostwald viscometer to obtain a ratio of flow times at the given temperatures. These were 0.8904 for SDS, 0.8800 for SDS/β-ME and 0.9988 for the buffer.

2.4. Sedimentation velocity measurements

Sedimentation velocity experiments were performed using an XLA (Beckman, USA) analytical ultracentrifuge equipped with scanning absorption optics. Experiments were carried out at 20°C and at rotor speeds between 15–40 000 rev/min. Absorption measurements were carried out at 280 nm. Ten consecutive scans were used to determine each sedimentation coefficient $s_{T,b}$. These values were converted to standard conditions (a temperature of 20°C and the viscosity of water as a solvent at 20°C) using the following equation [5];

$$S_{20,w} = \frac{(1 - \overline{v}\rho_o)_{20,w}\ \eta_{T,b}}{(1 - \overline{v}\rho_o)_{T,b}\ \eta_{20,w}} S_{T,b} \quad (2)$$

where $\overline{v}$ is the partial specific volume (ml/g) and ρ_o is the solvent density (g/ml).

2.5. Analysis

2.5.1. Molar mass

By combining the (z-average) diffusion coefficient from dynamic light scattering with the (weight average) sedimentation coefficient from the sedimentation velocity in the analytical ultracentrifugation, an apparent weight average [6] molar mass M_w could be estimated from the well known Svedberg Equation [7];

$$M_w = \frac{S_{20,w}\ RT}{D_{20,w}(1 - \overline{v}\rho_o)} \quad (3)$$

where R = gas constant ($JK^{-1}mol^{-1}$) and T = absolute temperature (K). It should be stressed that these are apparent molar masses, since the $s_{20,w}$ and $D_{20,w}$ values are not 'infinite dilution' values.

2.5.2. Gross Conformation

By 'double-log' plotting the ranges of values of $D_{20,w}$

and $s_{20,w}$ over the heating period against the calculated M_w the slopes of the lines can be taken as a function of conformation of the complex using the Mark-Houwink-Kuhn-Sakarada (MHKS) equations [8]:

Sedimentation coefficient (s);

$$s = K'' M^b \quad (4)$$

Diffusion coefficient (D);

$$D = K''' M^{-\epsilon} \quad (5)$$

The coefficients b and ϵ depend on the conformation [8].

3. Results and discussion

Heating 3% BSA at 95°C changed the pH (Fig. 1). In the absence of the reducing sugar there was an increase in the pH within the first 10 min and then values remained constant at approximately pH 7.0 for the next 70 min of heating. However, in the presence of xylose a pH fall occurred and the pH changed from 6.8 to a pH of less than 6. The fall occurred at a gradual and continual rate and was still decreasing when after 90 min of heating, gelation of the protein solution began. This pH fall may be a consequence of the alteration of the protein, e.g. removal of the basic ϵ-amino group of lysine and hence liberation of protons, and because of the formation of acidic Maillard reaction products. When heating was carried out at 121°C the fall was more severe with a pH of 5.0 being reached after 10 min [3].

The changes in the diffusion and sedimentation coefficients following heating of the protein are shown in Fig. 2. For BSA heated alone there was an initial decrease in the diffusion coefficient and rapid increase in the sedimentation coefficient during the first 10 min of heating. The values then remained approximately constant. This can be explained by the processes of denaturation and subsequent aggregation where the native protein molecules unfold on heating exposing hydrophobic regions which then promote association.

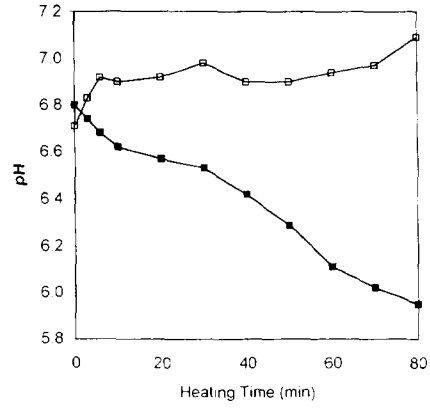

Fig. 1. Variation of the pH as a function of heating time at 95°C for 3% BSA (□) and 3% BSA/2% xylose (■).

When BSA was heated in the presence of xylose after 10 min the two parameters were essentially the same as observed for BSA alone. However, as heating proceeds there was a continuous and approximately linear decrease in the diffusion coefficient (Fig. 2a). Little difference was observed between the sedimentation coefficient values for the different systems (Fig. 2b).

The apparent molar masses obtained by combining the sedimentation and diffusion coefficients are shown in Fig. 3. For BSA alone the molar mass changed from a value of 125 kg/mol (denoting a dimer of the native BSA) to a value of 2000 kg/mol which indicates aggregates of approximately 30 BSA molecules. In contrast the calculated molar mass of BSA when heated in the presence of xylose continued to increase indefinitely throughout the heating period. From 20 to 45 min of heating the increase in molar mass was small, but at longer times the rate of increase became more rapid eventually resulting in gelation. This occurred when the pH decreased to 5.95.

An obvious question was whether this increase in molar mass observed for the BSA/xylose system was due to decrease in pH. To investigate this the pH of a BSA solution was lowered to 6.3, using 0.1 N HCl and heated at 95°C. Fig. 4 displays the pH and diffusion coefficient as a function of heating time. It can be seen that under these conditions the pH returns rapidly to near neutral. However, the decrease in the diffusion coefficient on heating was much greater than found for the xylose system even after this had obtained a pH as low as 5.9. Despite the small diffusion coefficient no gelation was observed for the pH 6.3 system. The diffusion coefficient should normally reflect the size and the extent of solvent associated with a molecule or aggregate of molecules. The very small diffusion coefficients obtained for the heated BSA originally at a low starting pH would indicate a highly aggregated system and/or a system which is highly expanded trapping a great deal of solvent.

To obtain information about the bonds maintaining the association products the heated protein was diluted

Table 1
Diffusion coefficient values for 3% BSA (initial pH 6.3) and 3% BSA/2% xylose after heating at 95°C
The BSA/xylose system was heated for 80 min and had resultant pH of 5.95. The lowered pH BSA was heated for 20 min and the pH at this time was 6.8. Samples were mixed in buffer (pH 6.8) plus sodium dodecyl sulphate (SDS) or SDS with beta mercaptoethanol (β-ME). Control experiments indicated that SDS did not interfere with the diffusion estimates.

System	(10^6) × Diffusion coefficient (cm^2/sec)		
	Buffer (pH 6.8)	SDS	SDS/β-ME
BSA (pH 6.3)	0.026 ± 0.001	0.05 ± 0.001	0.17 ± 0.002
BSA/xylose	0.12 ± 0.002	0.12 ± 0.003	0.13 ± 0.009

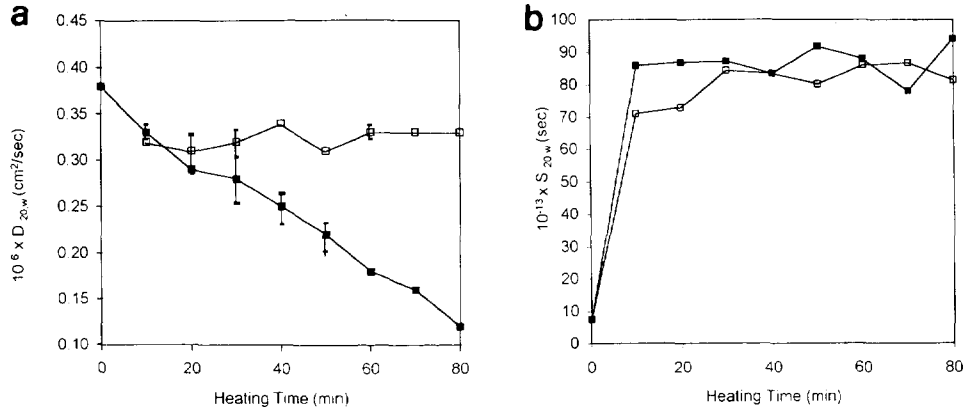

Fig. 2. Variation of (a) Diffusion coefficient and (b) sedimentation coefficient for 3% BSA/2% Xylose (■) and 3% BSA (□) as a function of heating time at 95°C.

in 1% SDS (to disrupt any hydrogen bonding and electrostatic interactions) or 1% SDS + 1% β-ME (to disrupt non-covalent linkages and disulphide bonds) prior to the measurement of the diffusion coefficient (see Table 1). In the case of the xylose system the results obtained were almost identical to those found when the diffusion coefficient was measured in native buffer. In contrast the protein which had aggregated by heating initially at a pH 6.3 gave diffusion coefficients in SDS and SDS + β-ME which were much higher than those found in the buffer. These results suggest that the aggregates formed in the xylose system involve non-disulphide covalent linkages resulting from the Maillard reaction.

It was possible to monitor the increasing size of the aggregates formed on heating BSA/xylose until the system gelled. Some information about the shape of these aggregates can be obtained from the double logarithmic plots of either sedimentation coefficient or diffusion coefficient against molar mass [8]. These plots are shown in Fig. 5, and the values obtained from the gradient of the slopes are:

$\epsilon = 0.92$
$b = 0.08$

These are close to the expected values for a rod ($\epsilon = 0.85$ and $b = 0.15$). This may suggest that the aggregates became larger by protein molecules associating in a linear (i.e. end to end) fashion. This is consistent with the so called 'string of beads' model originally proposed to explain the structure of protein gels [9]. It could be imagined that this organised aggregation would lead to fine stranded gels. A distinction can be made between fine stranded gels that form under conditions where electrostatic repulsion between molecules is moderately high and aggregated gels that form under conditions

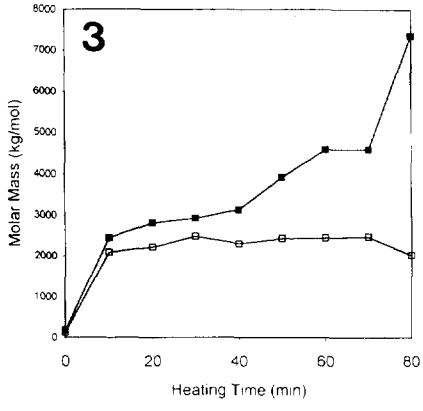

Fig. 3. Variation in Molar mass of 3% BSA/2% Xylose (■) and 3% BSA (□) as a function of heating time at 95°C.

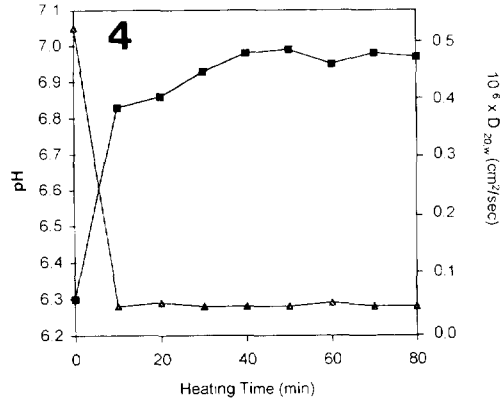

Fig. 4. Variation of Diffusion coefficient (▲) and pH (■) of low pH BSA with heating time at 95°C.

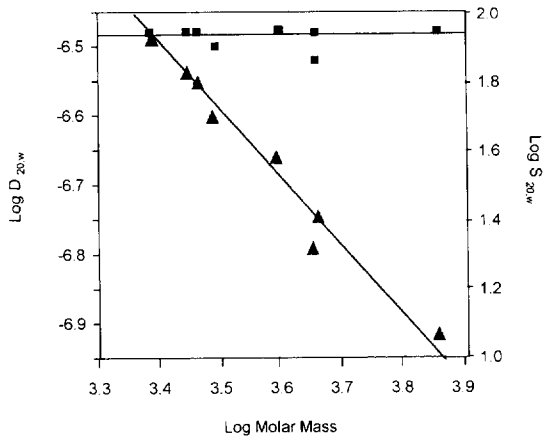

Fig. 5. Double logarithmic plot of sedimentation coefficient (■) and translational diffusion coefficient (▲) versus molar mass for a 3% BSA/2% xylose mixture.

closer to the protein isoelectric point and/ or high ionic strength [10]. It was previously suggested that Maillard gels are fine stranded in nature despite having a low pH [11]. The primary reason for this may be the enhanced net negative charge as a result of the loss of amino side chains from the protein [12]. This high charge would also explain why the diffusion coefficient of the xylose system is higher than that found for the system heated initially at pH 6.3 in the absence of the reducing sugar. Since it seems probable that the xylose system will have a higher net negative charge when the pH of the system is approximately 6 and thus non-covalent aggregation has not occurred. However, in the system lacking the reducing sugar aggregates are formed which the diffusion data indicates can be broken up by the inclusion of disrupting solvents.

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References

[1] Mauron, J. In: Maillard Reaction in Foods (C. Eriksson, ed.), Progress in Food Nutrition and Science, Pergamon, Oxford, 1981, 5, pp. 5–36.

[2] Monnier, V.M., Sell, D.R., Miyata, S. and Nagaraj, R.H. In: The Maillard Reaction, Advances in Life Sciences, Birkhauser Verlag Basel, 1990, pp. 393–414.

[3] Hill, S.E., Mitchell, J.R. and Armstrong, H.J. The production of heat stable gels at low protein concentrations by the use of the Maillard Reaction. In: Gums and Stabilisers in the Food Industry (G.O. Phillips, D.J. Wedlock and P.A. Williams, eds.), Elsevier Applied Science, 1992, pp. 471–478.

[4] Sanders, A.H. and Connell, D.S. In: Techniques for Light Scattering from Haemoglobin (V. Degiorgio, M. Corti and M. Giglio, eds.), Light Scattering in Liquids and Macromolecular Solutions, Plenum, New York, 1980, p. 173.

[5] Van Holde, K.E. Physical Biochemistry, Foundations of Modern Biochemistry Series, Prentice-Hall Inc., NJ. 1971.

[6] Pusey, P.N. In: Photon Correlation and Light Beating Spectroscopy (H.Z. Cummings and E.R. Pike, eds.), Plenum Press, New York, 1974, p 387.

[7] Tanford, C. Physical Chemistry of Macromolecules, Wiley, New York, 1961.

[8] Harding, S.E., Vårum, K.M., Stokke, B.T. and Smidsrød, O. In: Advances in Carbohydrate Analysis (C.A. White, ed.), JAI Press, Connecticut, USA, 1991, 1, pp. 63–144.

[9] Tombs, M.P. Alterations to proteins during processing and the formation of structures. In: Proteins as Human Food (R.A. Lawrie, ed.), Butterworth and Co Ltd., 1970, pp. 126–138.

[10] Langton, M and Hermansson, A,-M. Fine stranded and particulate gels of β-Lactoglobulin and whey protein at varying pH. *Food Hydrocolloids* 1992; 5: 523–539.

[11] Armstrong, H.J., Hill, S.E., Schrooyen, P. and Mitchell, J.R. A comparison of the viscoelastic properties of conventional and Maillard protein gels. *J. Texture Stud.* 1994; 25: 285–298.

[12] Yaylayan, V.A., Huyghues-Despointes, A. and Polydorides, A. A fluorescamine-based assay for the degree of glycation in Bovine Serum Albumin. *Food Res. Int.*, 1992; 25: 269–275.