

EFFECT OF ASCORBIC ACID, DIKETOGULONIC ACID AND DEHYDROASCORBIC ACID ON BOVINE SERUM ALBUMIN ON STORAGE IN THE GLASSY STATE*

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Abstract – Showing the occurrence of glass/rubber transition in bovine serum albumin (a globular protein) at low moisture levels and the occurrence of Maillard crosslinking in gelatin in previous works, the importance of the state of glass or rubber of BSA on the rate and extent of polymerisation, protein solubility, colour and SDS PAGE patterns in the presence and absence of ascorbic acid, diketogulonic acid and dehydroascorbic acid was investigated. BSA on storage with Ascorbic acid and its degradation products at low moisture contents (8.3% and 19.1%) at a relatively low temperature (40°C) can undergo chemical changes resulting in marked increase in molecular weight, solubility decrease and formation of browning colour. The glassy state data established the occurrence of chemical changes (Maillard crosslinking and disulfide crosslinking) in the BSA system on storage below its glass transition temperature.

Keywords – Bovine serum albumin, glass transition, ascorbic acid, crosslinking, low moisture

1. INTRODUCTION

Most raw materials and many half processed and finished products of food and pharmaceutical industries are stored in glassy state and low moisture contents for times ranging from days to years before subsequent processing or consumption. The mobility of molecules is greatly reduced in the glassy state resulting in an increase in the stability of synthetic and biological systems through the significant decrease of the rate constant of chemical and physical processes [1-6].

Molecular relaxation processes may still take place in the temperature range below the glass transition known as β -relaxations with a lower amplitude compared to the molecular relaxations above the T_g (α -relaxations). Therefore, limited movement of atoms, molecules or side chains of food polymers within the glassy state may still occur, and this may lead to chemical or physical changes that affect food quality [5-8].

Bovine serum albumin is a non-glycoprotein characterized by low content tryptophane and methionine, and a high content of cysteine (odd number) and lysine, and contains 0.3-0.5 mole of fatty acids per mole. BSA may contain 4-10% of dimers and higher oligomers, and the molecular weight of defatted monomeric BSA is about 67 kDa. Monomeric forms of BSA are believed to be more stable in dilute solutions, ~10 g/liter than as powders, since lyophilization can promote polymer formation [9].

Covalent crosslinking can have a profound effect on molecular weight, structure and functions of proteins in food and pharmaceutical products [6, 8, 10, 11]. These changes may cause a deterioration in food quality or affect drug stability on storage. Ascorbic acid and its degradation products are capable of crosslinking the proteins and producing red pigment in dilute solutions [12-15].

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Farahnaky and co-workers [16] indicated that ascorbic acid can crosslink gelatin chains and decrease its solubility at low moisture contents. Glass rubber transition has been seen on a DSC thermogram of BSA at low moistures (3-19% moisture wb), proving that the idea of T_g can also be extended to the storage of globular proteins. Endothermic peaks that corresponded to enthalpy relaxation were observed for commercial samples of bovine serum albumin (BSA) showing the occurrence of physical ageing in a globular protein [17].

The objective of this study was to investigate the idea of crosslinking by ascorbic acid and some of its degradation compounds and evaluate the induced changes occurring in a globular protein model system on storage in the glassy state at low moisture contents at a temperature that could be applicable to the accelerated storage trials of materials. For this purpose, the effects of ascorbic acid and its products on bovine serum albumin (BSA) in the glassy state at low moisture contents (<20%) at 40°C were studied.

2. MATERIALS AND METHODS

Bovine serum albumin, electrophoresis grade, (catalogue number A-7906, lot 81K1813), was purchased from Sigma. Ascorbic acid (AA), 2,3-diketgulonic acid (DKG), dehydroascorbic acid (DHA) and all other chemicals were of analytical grade and purchased from Sigma Co. or Fisher Co. (Loughborough, UK).

a) Sample preparation

Solutions of BSA (30%) with 1% ascorbic acid, 1% dehydroascorbic acid (reduced form of ascorbic acid) or 1% 2,3-diketgulonic acid were prepared by dissolving the protein in distilled water at ambient temperature and mixing for 10 min using a magnet stirrer. The pH of the solutions was about 5.5. These solutions were then freeze-dried and stored at 40°C at relative humidities of 53% or 82% for 6 weeks. A BSA sample without AA, DHA or diketogulonic acid was used as a control. All tests were performed at least in triplicate.

b) Protein solubility

The solubilities of BSA samples in water and aqueous solutions of 1% sodium-dodecyl sulphate (SDS) or 1% SDS + 1% β-mercaptoethanol (βME) were measured. An aliquot of each sample (0.1 g) was mixed with 10 ml of each solvent (water and SDS) and shaken overnight in a flask shaker. The samples were then centrifuged for 10 min at 1500 g and filtered through Whatman No. 4 paper. An aliquot of each supernatant was assayed for protein by an adoption of the Lowry method [18]. Bovine serum albumin without any treatments was used as a standard to plot the calibration curve [19].

c) Color measurement

Colour measurements of BSA samples (sieved through a sieve of 120 micrometer) were carried out using a Colourquest spectrophotometer SN C 5330 (Virginia, USA). Parameters of a, b, and L of the samples were measured.

d) Average molecular weight determination

To monitor molecular weight (M_w) changes in the BSA samples, size exclusion chromatography multi angle laser light scattering (SEC/MALLS) was used [20]. The SEC/MALLS consisted of a degasser (Degassy DG-1200, HPLC Technology, Macclesfield, UK), a solvent delivery system (Model 590, Waters, Millipore, Watford, UK), a Rheodyne model 7125 injection valve (Rheodyne Inc., Cotati, USA) fitted with a 100 μL loop, and PSS Hema Bio Linear and PSS Hema Bio40 columns (manufactured by PSS GmbH, Mainz, Germany). The eluent (deionised water) was pumped at a flow rate of 0.8 ml/min and

the injection volume was 100 μL . Column effluent was monitored using a Dawn F laser light scattering photometer (Wyatt Technology, Santa Barbara, USA) fitted with a 5 mW He-Ne Laser and an Optilab 901 interferometric refractometer (Wyatt Technology, USA). The protein samples were dissolved in a 1% SDS aqueous solution before injection into the system. As the samples stored at an RH of 82% contained insoluble parts, for M_w assessment the soluble fractions as defined by the method used for solubility in 1%SDS solution were used.

e) SDS-PAGE

Gradient SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed according to the discontinuous buffer system described by Laemmli [21] using precast gels (4-15%) from BIORAD. The protein samples were dissolved in a sample buffer including 10% SDS and 1.2% DTT (Dithiothreitol) for breaking non-covalent and disulphide bonds. Electrophoresis was carried out at a constant current of 100 mA and ambient temperature. Proteins were visualised using Coomassie Brilliant Blue R-350 (Phast Gel Blue R, Amersham Pharmacia Biotech).

f) Differential scanning calorimetry

A DSC-7 (Perkin-Elmer, Beaconsfield, UK), calibrated with indium and cyclohexan, was used to analyse BSA samples (10-15 mg) held at RHs of 53 and 82% for 2 weeks at 40°C for equilibrium. High pressure stainless steel pans were scanned at a heating rate of 10°C. min⁻¹, from -20 to 135°C. After cooling (50°C/min) the samples were heated once again using the same conditions. An empty stainless steel pan was used as reference.

g) Moisture content determination

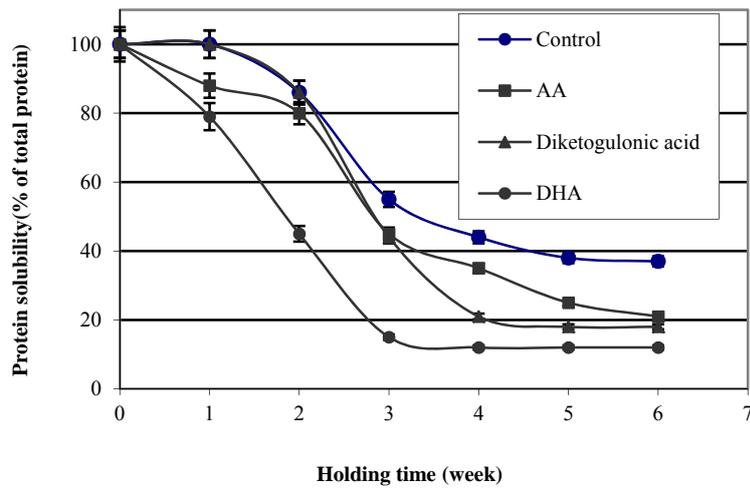
Moisture content of the samples was determined by oven drying at 100°C until a constant weight (about 6 hours).

h) Statistical analysis

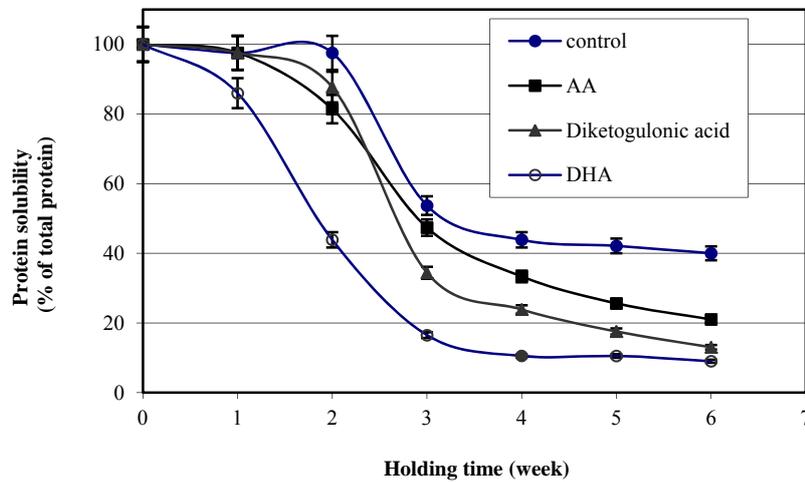
To compare the average values of each parameter measured, the multiple range Duncan's test was used.

3. RESULTS

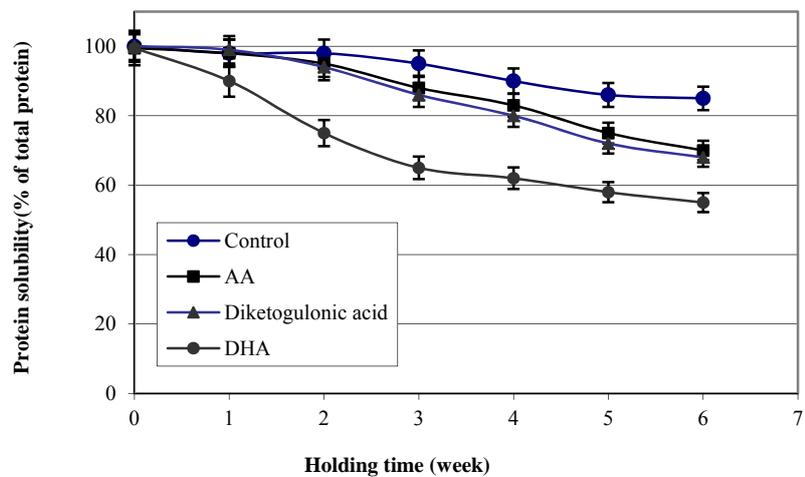
Solubility changes of the BSA samples on storage with 1%AA, DHA or DKG at an RH of 82% are shown in Figs. 1(a), 1(b) and 1(c). AA, DHA and DKG significantly lowered the solubility of the BSA samples in aqueous solutions including 1%SDS or 1%SDS+1%BME, compared to the controls. After six weeks of storing BSA samples at an RH of 82%, solubility losses of control and the BSA samples with AA, DKG and DHA in 1% SDS were about 60, 80, 85 and 90% of total protein, respectively, while these values for solubility losses in 1% SDS+1% BME were about 15, 30, 30 and 45% of total protein. However, the storage of the BSA samples with AA, DHA and DKG for 6 weeks at an RH of 53% at 40°C did not decrease the protein solubility in 1%SDS or 1%SDS+1%BME except for DHA which, after 6 weeks, a solubility decrease of around 10% was observed, while no solubility decrease was found for the controls.



(a)



(b)



(c)

Fig. 1. Effect of AA (1%), DHA (1%) and diketogulonic acid (1%) on solubility of BSA held at RH of 82% in (a) water (b) 1%SDS or (c)1%SDS+1% BME aqueous solutions. The values are average of 3 replicates.

Figs. 2 and 3 show the average molecular weight changes of BSA samples stored at RHs of 82 and 53% at 40 °C for 6 weeks. At 82% RH there was a drastic difference between the average molecular weights of the control and the samples with 1%AA, DHA or DKG. After two weeks, M_w of the samples with AA, DHA or DKG increased to about 2,500,000 (g/mol) and remained at the same level up to 6 weeks of storage. At an RH of 53%, again there was a marked difference between the samples with AA, DHA and DKG and the control, while the molecular weights obtained for the BSA samples with AA, DHA, and DKG were several times higher than that of the control (Fig. 3).

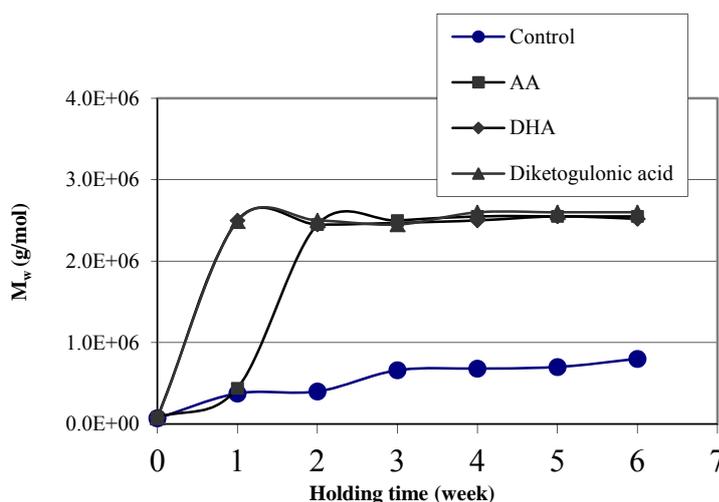


Fig. 2. Average molecular weights of BSA samples stored with 1%AA or 1% diketogulonic acid or 1%DHA for 6 weeks at 40°C at RH of 82% as determined by SEC/MALLS

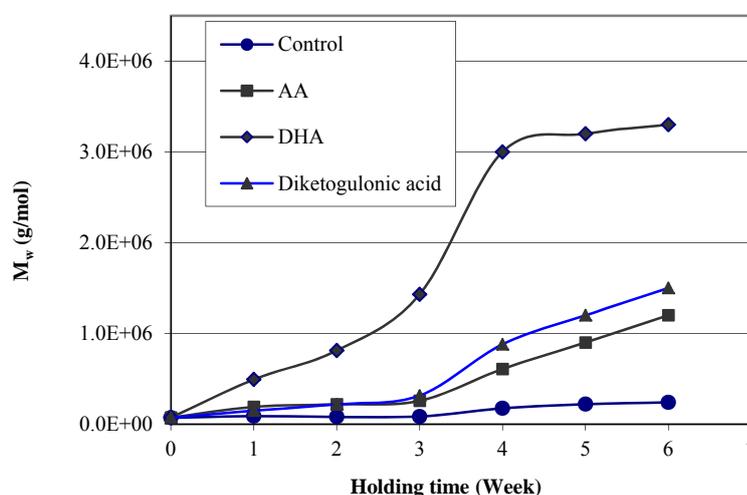


Fig. 3. Average molecular weights of BSA samples stored with 1%AA or 1% diketogulonic acid or 1% DHA for 6 weeks at 40°C at RH of 53%

SDS-PAGE patterns of BSA with 1% AA (Fig. 4(a)), 1% DKG (Fig. 4(b)) or 1% DHA (Fig. 4(c)) held at 40°C from 1 to 4 weeks at an RH of 53% show that, for the controls the major part of the BSA molecules appeared at the regions between 66 kDa and 220 kDa, while for the samples with AA, DHA and DKG the intensity of the bands around 66 kDa decreased and the intensity of the bands with higher M_w (>97 kDa) increased. For BSA samples stored at an RH of 83%, large protein aggregates were found to form as early as one week (for samples with DHA or DKG) or two weeks (for samples with AA) that did not enter the polyacrylamide gels.

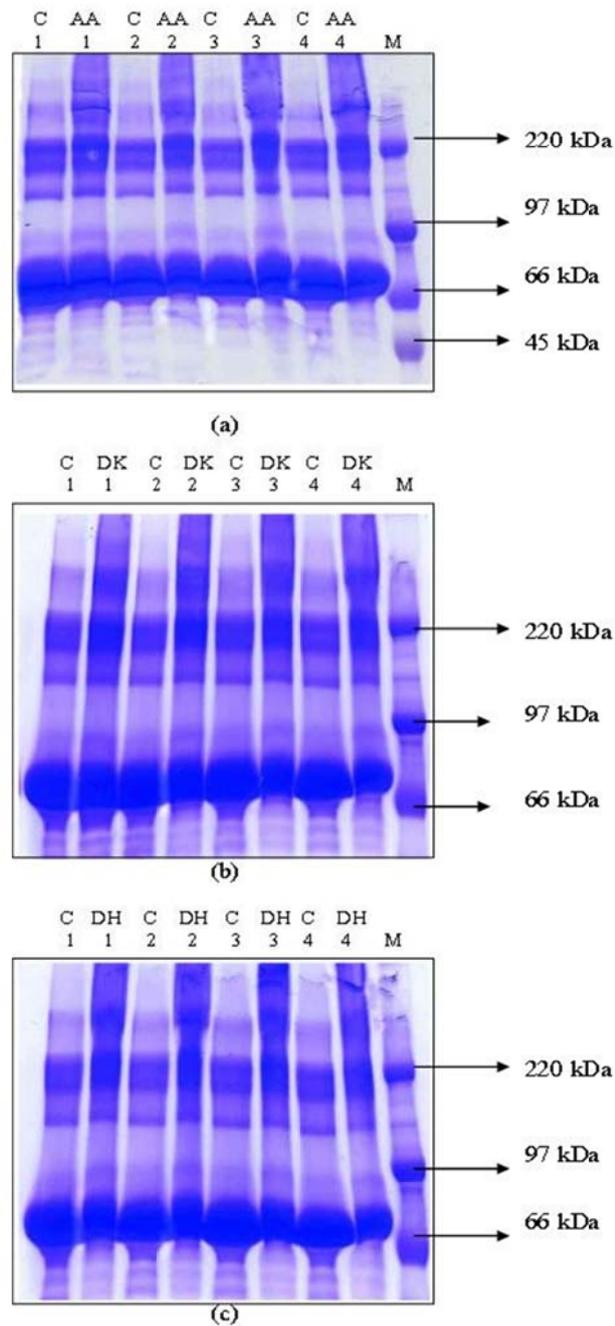
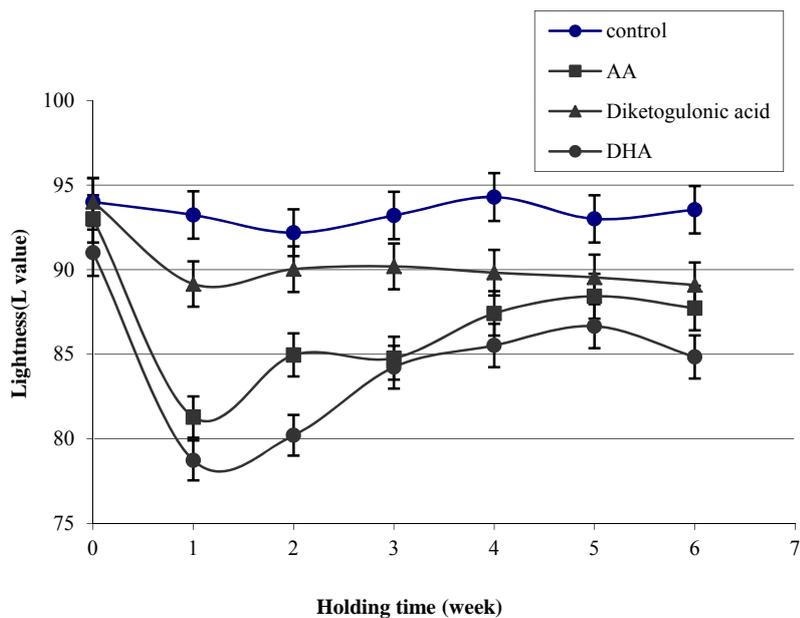


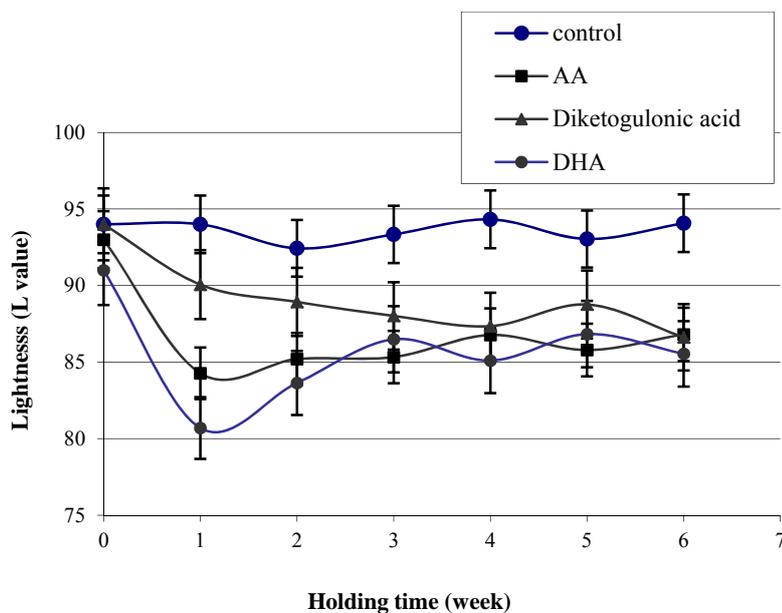
Fig. 4. SDS-PAGE patterns of BSA with 1% ascorbic acid (a), 1% diketogulonic acid (b) or 1% dehydroascorbic acid (c) held at 40 °C from 1 to 4 weeks at RH of 53%. (The numbers on top indicate the week (weeks) of storage of the samples, C (control), AA (ascorbic acid), DK (Diketogulonic acid) and DH (dehydroascorbic acid), M (molecule weight marker))

The effect of AA, DHA and DKG on the lightness of BSA samples on storage at RHs of 82 and 53% is shown in Fig. 5. Having a difference of 1 unit in the lightness value between the two samples can be detected by human eyes. These additives significantly increased the darkness of the BSA samples held at 40°C for both RHs compared to their controls.

Differential scanning calorimetry data (Table 1 and Fig. 6) show that BSA samples stored at an RH of 53% and 40°C with 8.3% moisture were in the glass, while the samples stored at 82% RH with 19.1% moisture were in the conditions which present a point between the glass and rubber.



(1)



(2)

Fig. 5. Effect of ascorbic acid, diketogulonic acid and dehydroascorbic acid on the lightness of BSA held at 40°C at RHs of 53% (1) and 82% (2) for 6 weeks. Bars are ±1SD

Table 1. Glass transition temperature and moisture content of BSA samples stored at RHs of 53% and 82%. Moisture content and glass transition values are the average of 3 and 2 replicates, respectively

	RH of 53%	RH of 82%
Moisture content (wb)	8.3%	19.1%
Transition onset temperature	82 °C	30°C
Transition endset temperature	108 °C	85°C
Half Cp extrapolated	96 °C	57°C
Peak 1 (Max)	74 °C	72°C
Peak 2 (Max)	115 °C	92°C

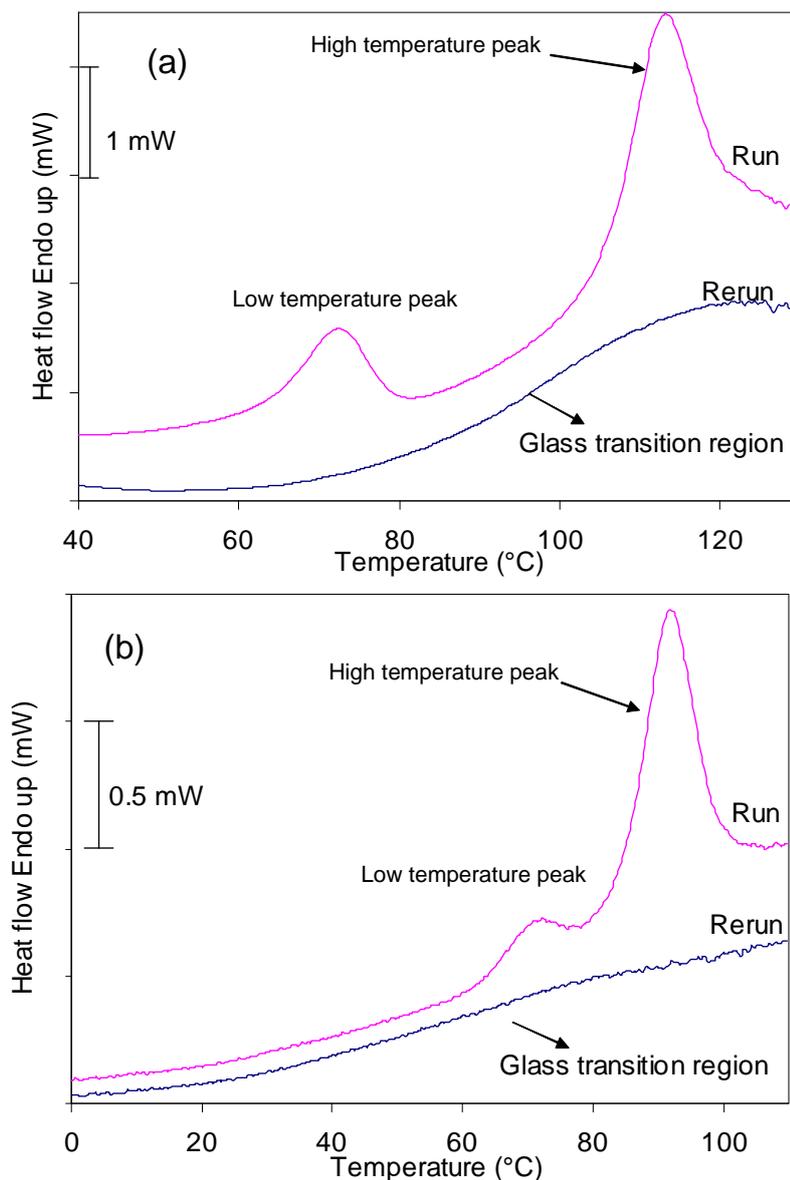


Fig. 6. DSC scans for the BSA sample equilibrated at 40°C (a) RH of 53% (moisture content 8.3% wb), (b) RH of 82% (moisture content 19.1% wb)

4. DISCUSSION

Comparison of solubility losses of the samples stored at an RH of 83% in aqueous solutions including 1% SDS or 1% SDS+1% BME showed that at low moisture contents the aggregation of BSA molecules may occur through intermolecular disulfide bonds. Other mechanisms such as protein oxidation and denaturation due to covalent and non-covalent bond are also possible. The marked decrease observed in the solubility of the BSA control was in good agreement with Champion and co-workers [7] who reported the solubility loss of a variety of BSA samples on storage at 37°C and an RH of 96%. Significant differences in the solubility of BSA samples with AA, DHA or DKG in 1%SDS+1%BME compared to the control showed that some non-hydrogen and non-disulfide bonds were involved to make the BSA samples insoluble. These solubility changes were not observed for RH of 53%, except for the BSA sample with DHA stored for 6 weeks, showing the importance of the state of glass or rubber of the materials and

the moisture content on the rate of reactions in the system. The order of crosslinking reactivity found was DHA > AA and DKG.

Although little change was seen in the solubility of the BSA samples when they were stored in the glass (BSA samples held at 53% RH), more significant changes were recorded in average molecular weights, SDS patterns and color. This could be related to β -relaxations and the mobility of side chains or the diffusion of small molecules in the system, while the main back bone has limited mobility.

Ascorbic acid is relatively labile and degrades during processing and storage of foods to give a large number of products such as DHA, 2,3-diketogulonic acid, glyoxal, methylglyoxal, threose and oxalic acid [3, 5]. Ortwerth and co-workers [22] found that ascorbic acid can crosslink lens proteins and decrease their solubility, and lysine was the major amino acid modified by ascorbic acid. Ribonuclease A model protein can be crosslinked in the presence of DHA or its degradation products. When all of the amino groups were selectively protected with succinic anhydride, no crosslinking was observed by DHA and the reaction of oxalic acid (carboxylic acid) with RNase A did not result in the formation of protein crosslinks, proving the Maillard type mechanism (carbonyl and amino groups) was involved. Lysine was found to be the most reactive amino acid [15].

In conclusion, BSA on storage with AA and its degradation products at low moisture contents (8.3% and 19.1%) at a relatively low temperature (40°C) can undergo chemical changes resulting in a marked increase in M_w , solubility decrease and formation of brown color. These changes can occur even when the protein was stored at the glassy state. Many foods and pharmaceutical products stored in the glassy state are therefore not fully protected against reactions that lead to quality deterioration. The overall picture that appears to emerge from the present study is that there may be significantly more molecular mobility in the glassy materials than might previously have been expected. Such results would be in agreement with Schebor and co-workers [6] who stated that T_g cannot be considered as an absolute threshold of stability.

The recorded effects of ascorbic acid and other chemicals tested in this research on protein crosslinking can have practical implications for the stability of many foods and pharmaceuticals on storage at low moisture contents.

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