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An Investigation on Physical Ageing of β-Lactoglobulin and Implications for Its Storage

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Abstract

Due to the importance of physical ageing in functional properties of the globular protein of β-lactoglobulin as the main protein of whey powder (the by product of cheese manufacturing industry) the possible occurrence of physical ageing in dry powder of β-lactoglobulin with moisture content of 15.9% was studied at different temperatures below and close to the glass transition.

Endothermic peaks that corresponded to relaxation enthalpy were observed for a β-lactoglobulin with 4 % moisture. Enthalpy and peak temperature increased on storage of β-lactoglobulin when it was held in the glassy state at different temperatures (20, 40, 60 º C). The general characteristics of the endothermic peaks were similar to those previously reported for synthetic polymers.

KEYWORDS: B-lactoglobulin, physical ageing, DSC

1.Introduction

Physical ageing occurs universally in the glassy state irrespective of the chemical nature of a polymer. Physical ageing has been seen in a large variety of materials, including fibers, biopolymers and synthetic polymers. Physical ageing is a well-known phenomenon in polymer glasses and occurs when a polymer is rapidly cooled from the equilibrium rubber or liquid state into the non-equilibrium glassy state. This glass will gradually relax towards equilibrium. The relaxation of the polymer manifests itself as changes in its mechanical properties, such as density, brittleness and also dielectric loss. A change in the thermodynamic properties, such as enthalpy, specific volume and refractive index, also occurs. A convenient measure of the amount of physical ageing is to measure the enthalpy relaxation by differential scanning calorimetery (DSC). A decrease in enthalpy as a result of the relaxation process can be analyzed as an enthalpy recovery peak (or 'overshoot') during a heating DSC scan. The intensity and the position of the enthalpy recovery peak depend on both the nature of the sample and how the sample was prepared, i.e. its thermal history (Matveev and Grinberg 1997, Wungtanagorn and Schmidt 2001, Farahnaky and others 2005).

Physical ageing has implications for the stability and conservation of performance for many materials stored at low moisture contents. This phenomenon has been investigated and reported on over the last 60 years for synthetic and inorganic polymers and now its implications for pharmaceuticals and food materials are actively being investigated. Most of research on physical ageing of biological materials has focused on carbohydrates such as starch, starch based products and sugars e.g. Physical aging of amorphous anhydrous fructose at temperature 5 °C and at 22 °C was studied using differential scanning calorimetry (DSC) by Truong and others (2006). However there are a limited number of reports on physical ageing of proteins (Martinet 2001).

Whey, a liquid by-product of the cheese manufacturing process, is normally converted for practical use to a powder within a spray dryer. This powder is comprised of approximately 75% lactose, 22% protein and salts, and a remainder consisting of lactic acid and fat. β-Lactoglobulin is the most abundant protein in bovine whey, comprising almost 50% of the total protein; therefore, its properties are very important to the functionality of milk and isolated whey proteins (Nijdam and others 2008). β-Lactoglobulin is a water soluble, globular protein of 18,300 Da, corresponding to a polypeptide of 162 residues for all genetic variants. At physiological pH it is a dimmer, but dissociates into monomers below pH 3.0. It has five cysteine residues, three of which form two possible disulfide bonds; the unusual properties of these labile cysteines influence many of its functionalities in foods. β -Lactoglobulin has a protein fold composed of two antiparallel beta-sheets (Sundaresan and others 2008).

Whey powder as a glassy matter may be stored for weeks and even months during transportation and marketing before being used in food and no-food products. Storage of whey powder could cause some physiochemical changes resulting in deterioration of its functional properties such as solubility, emulsifying ability and water interaction. Physical ageing has implications for the stability and conservation of performance for many materials stored at low moisture contents. The possible occurrence of physical ageing in β-lactoglobulin as the main protein of whey powder may increase the loss of its solubility and other useful properties.

The aim of this current study was to explore the occurrence of physical ageing in β-lactoglobulin and determine the extent of its structural relaxation.

2.Experimental

Materials

β-lactoglobulin (minimum purity of 99%) was purchased from Sigma-Aldrich (Poole, UK) (L0130) and is a mixture of genetic variants A and B with the moisture content of 4% (dry basis). For physical ageing study β-lactoglobulin equilibrated over supersaturated solution of NaBr with the moisture content of 15.9% was used.

All chemicals used in this research were of analytical grade unless otherwise mentioned.

Structural relaxation using DSC

A DSC-7 (Perkin-Elmer, Beaconsfield, UK), calibrated with indium and cyclohexane, was used to analyze β-lactoglobulin samples (10-15 mg). High pressure stainless steel pans containing β-lactoglobulin samples were scanned at a heating rate of 10 $K.min^{-1}$, from 0 to 100 °C except for the DSC test on sample as received which 0-160 °C was used. After cooling (50°C/min) the samples were heated once again using the same conditions to measure their glass transition temperatures.

For physical ageing study after eliminating the thermal history of β-lactoglobulin by heating in the DSC, the sealed pans containing β-lactoglobulin samples were held at different temperatures (20, 40 and 60 ºC) for up to 24 hours and rerun in the DSC to explore possible physical ageing phenomenon. An empty stainless steel pan was used as reference. Pyris software (Perkin-Elmer) was used to analyze the DSC traces.

Protein solubility

Protein solubility of the samples before and after storage at different temperatures in water, 1% sodium-dodecyl sulphate (SDS) or 1%SDS + 1%β-mercaptoethanol was measured by an adaptation of the Lowry method. An aliquot of each sample (0.1 g) was mixed with 10 ml of each solvent and shaken overnight in a flask shaker at ambient temperature. The samples were then centrifuged for 10 min at 1500 g and filtered through Whatman No. 4 paper and the supernatant assessed for protein concentration (Paterson, 1979).

3. Results and discussion

It is generally easy to see the glass transition (Tg) in non-globular proteins, but this is not the case for native globular proteins. Only after the globular proteins are hydrated may an exceptionally broad or stretched out transition be observed by DSC and rheological techniques. As glass transition peaks can occur as the denaturation of the protein begins it can be difficult to differentiate between the reversible and non reversible event. Hence, glass transitions are easier to identify when the protein has lost some of its tertiary order (Rupley and Careri 1991, Sartor and others 1994, Ferrari and Johari 1997, Tsereteli and others 2000, Brownsey and others 2003).

Although DSC technique has been capable of providing traces with clear glass/rubber transition for many proteins at low moisture contents in particular for gelatin, gluten and globular protein of BSA (Farahnaky and others 2008), the delta heat capacity of β-lactoglobulin at glass rubber transition region was low therefore Tg was less clear. The analysis of the data obtained showed that Tg of the β-lactoglobulin used for physical ageing was about 80 ˚C. Therefore for physical ageing study the samples were aged at 20, 40 and 60 ˚C.

The β–lactoglobulin (4% moisture) obtained from Sigma without any modification was scanned by differential scanning calorimetry from 10 to 160°C. The DSC traces are shown in Figure 1. Although on arrival the sample had been stored at 4°C in sealed containers, its overall thermal history after production and drying (after entering into the glassy state) was unknown. On the first run two endothermic peaks are observed. One peak appears at about 20-65 °C and other peak appears at about 95- 150 °C. The high temperature peak was thought to be due to protein denaturation as did not reappear on the second run DSC scan even after storage. The origins of the low temperature peak need to be considered. Bimodal peaks for globular proteins have been reported and are said to be due to biphasic denaturation and this can be more evident in samples containing fatty acids (Michnik 2003). However, the peak reappearance at low temperatures on storage of dry β-lactoglobulin at 20, 40 and 60 °C confirmed that the low temperature peak was not related to denaturation and is more likely to be due to physical ageing.

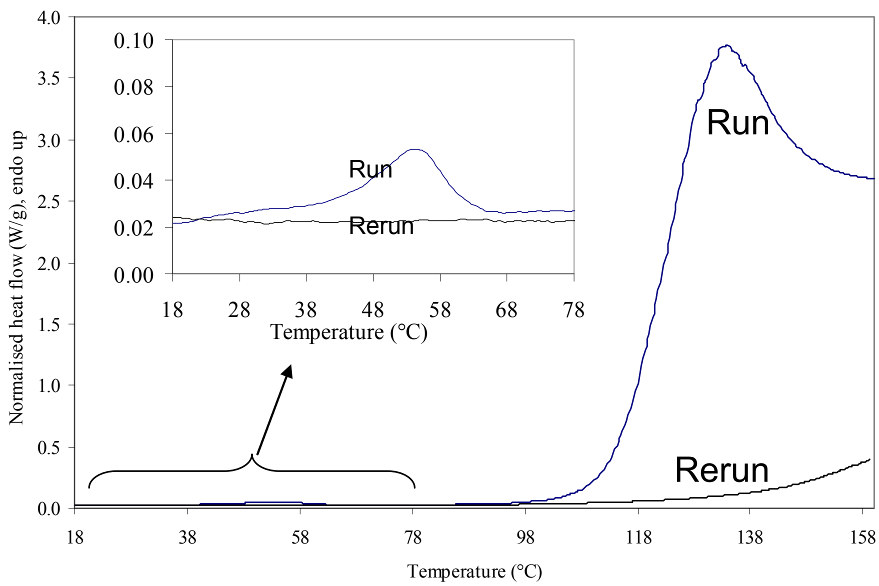

Figure 1. Differential scanning calorimetry trace of β-Lactoglobulin as received from Sigma with the moisture content of 4%.

For physical ageing study as thermal history and cooling rate of a glassy polymer affects the rate at which a system reaches equilibrium, it is essential to prepare the samples with similar thermal histories. The β-lactoglobulin samples with moisture content of 15.9% were sealed in the DSC pans and heated in the DSC up to 100ºC at 10ºC/min and cooled down at 50ºC/min. The sealed β-lactoglobulin pans were then stored for different times ranging from 1 to 24 hour at three temperatures 20, 40 and 60 ºC. These temperatures were used to represent a range of temperatures below the glass transition temperature of the β-lactoglobulin samples. After storage, the pans were rerun in the DSC and the thermograms were obtained. After subtracting the DSC trace of each sample before and after storage, as seen in Figure 2, there is a low temperature peak that increases with the ageing time and ageing temperature. When amorphous polymers are stored below their glass transition temperatures, a spontaneous decrease in volume or enthalpy known as physical ageing is observed. On storage there is a relaxation towards the equilibrium state. This enthalpy loss is regained on heating and can be observed as an endotherm appearing on the DSC thermogram (Montserrat 1994, Thiewes and Steeneken, 1997). It is therefore possible that the peak being observed corresponds to physical ageing.

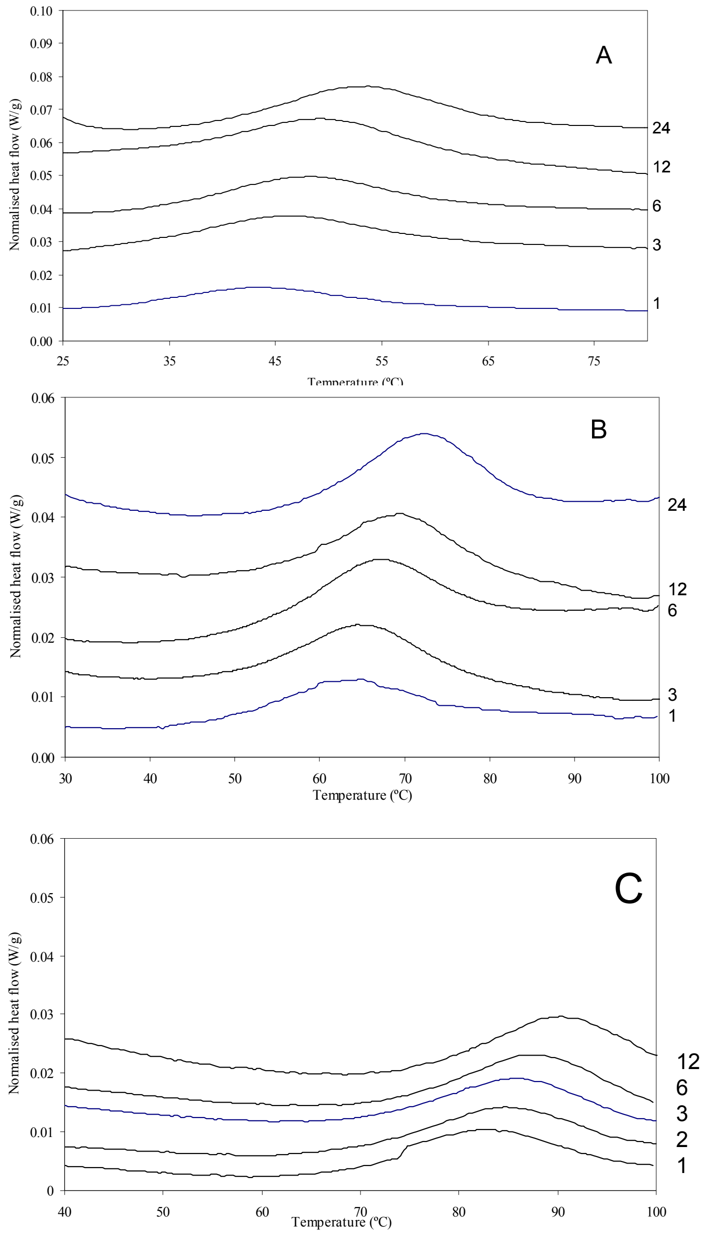

Figure 2. DSC curves of β-lactoglobulin samples aged at 20(A), 40(B), and 60 (C) ºC for up to 24 hours. The growth of an endothermic peak with storage time can be seen in the temperature range of 30-75, 40-90 and 65-100 ºC for storage temperatures of 20 and 40 and 60 ºC, respectively. The numbers next to each curve show the storage time in hours.

Plots of peak temperature versus logarithm of ageing time (min) are shown in Figure 3 for the β-lactoglobulin samples aged at 20, 40 and 60 ºC. There is a linear dependence between the peak temperature and the logarithm of ageing time, this is a well-reported characteristic of physical ageing of synthetic polymers and is in agreement with the findings on starch and starch sugar systems (Martinet 2001). The storage temperature does not seem to influence the slope. The temperature at which the endothermic peaks appear depends on the storage temperature as is shown in Figure 3, the higher the storage temperature the higher the peak temperature of the endotherm. For all three ageing temperatures, after 3 hours of ageing, the peak temperatures appeared approximately 25 ºC above the temperature of storage. This was similar to the previous findings on bovine serum albumin and red crayfish proteins reported by Farahnaky and others (2005, 2008).

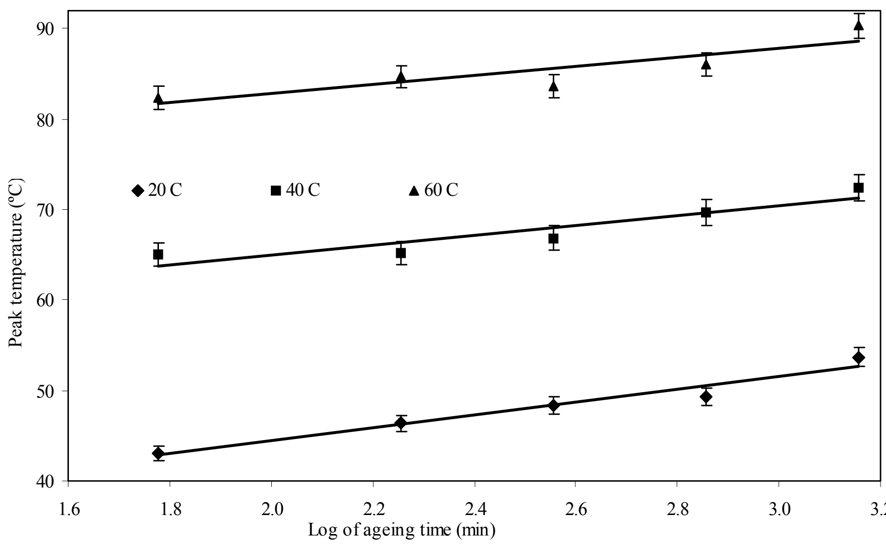

Figure 3. Dependence of the peak temperature on the logarithm of the ageing time (min) for β-lactoglobulin samples aged at 20, 40 and 60 ºC. Each bar is ±SD.

The plot of enthalpy versus log of ageing time in Figure 4 reveals that enthalpy increases linearly with log of ageing time, and this is a well reported typical characteristic of polymers during physical ageing process.

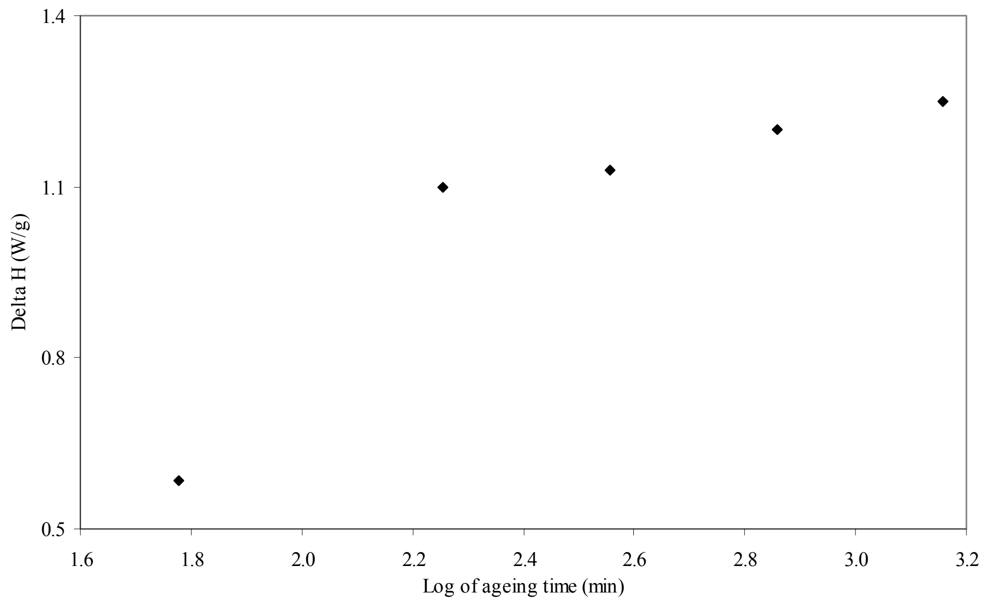

Figure 4. Ageing endothermic energy of β-Lactoglobulin sample containing 15.6 % water after storage at temperatures of 25 ºC.

Although some chemical change or relocation of water or other physical change may explain the results, the position and the growth dependency of the low temperature peak does resemble those known as enthalpy relaxation for other polymers.

4. Conclusions

An endothermic peak was seen for β-lactoglobulin occurring in the region below the observed Tg and the properties of these peaks were similar to those reported for endothermic peaks derived from physically aged synthetic polymers such as poly (vinyl pyrrolidone) and poly (methyl methacrylate) (Kalichevsky and Jaroszkiewicz 1992, Hancock and others 1995).

The results from this study would suggest that β-lactoglobulin undergoes substantial physical ageing, and it is worth considering why this could be so. The flexibility of the protein molecules would be low at a molecular level, as so much of the backbone is organized into higher orders of structure. Mobility and change of side chains could be possible even when the backbone of the molecule is in the glass. This local movement could explain the changes in the glass.

Physical ageing relates to a loss of volume of the polymer and its densification. On drying proteins molecules undergo compression of the globule and 4-6% of their volume is lost. Therefore change and compression of molecular structures does occur. Storage of β-lactoglobulin samples at 25, 40 and 60 ºC up to 24 hours did not cause any decline in protein solubility. Although protein solubility did not alter as affected by storage, solubilization of physically aged samples took longer compare to the control sample.

The findings of this study would be beneficial for optimizing the storage conditions of β-lactoglobulin and whey powder. These could also have practical implications for better use of maximum functionality of β-lactoglobulin as the main protein of whey powder with its expanding market, therefore requires further studies.

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