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Research paper

The aggregative stability of β-lactoglobulin in glassy mixtures with sucrose, trehalose and dextran

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ABSTRACT

The aim of this study was to investigate the effect of the addition of different carbohydrates on the thermally induced aggregation of a model globular protein, β-lactoglobulin (BLG), in the glass state. Amorphous mixtures of BLG with trehalose, sucrose and dextran were prepared by freeze-drying, their glass behaviour was characterised using calorimetry and thermally induced aggregation was measured using size exclusion chromatography. Pure BLG shows increasing levels of aggregation when heated in the temperature range 70–100 °C for 48–144 h. The addition of the disaccharides sucrose and trehalose both resulted in a decrease in aggregation rate which approached negligible rates at 50 wt.% carbohydrate. The effect of dextran addition was similar to that of the disaccharides when preparations containing 9 wt.% carbohydrate were heated at 70 °C for 2 days. However, when the concentration exceeded 23 wt.%, the reaction temperature was 70 °C or above or the reaction time was longer than 48 h, the addition of the polysaccharide did not protect the protein from thermally induced aggregation, suggesting that protein–polymer phase separation could have occurred during freeze-drying. Overall the results support the proposal that one aspect of carbohydrate additive functionality is as a diluent with the added condition that the carbohydrate remains miscible with the protein during processing.

1. Introduction

Biopharmaceuticals are commonly stabilized by being dried into an amorphous glass state in the presence of stabilizing carbohydrate additives [1,2]. Freeze-drying and spray-drying are commonly used technologies to prepare biopharmaceutical glasses. Freeze-drying, though expensive, has the advantage that thermally activated processes are minimised by taking a low temperature processing route [1,3]. Mixed protein/carbohydrate glasses have a variety of useful functional properties. In addition to increasing protein stability at ambient and sub-ambient temperatures, they also increase stability at elevated temperatures as in antiviral dry heat processing of proteins [4] and, as a solid they are a convenient state for the preparation of particles as in the fabrication of inhalable formulations. While glass state technologies offer undoubted benefits, some of the processing steps can impact on the structure and integrity of the proteins, like degradative changes induced in protein formulations caused by the freezing and drying steps of the freeze-drying process [3–5]. Although many aspects of the mechanisms of glass state stabilization technology are becoming well understood, a wholly coherent description of both processing and subsequent stabilization is yet to emerge [1,5,6].

One reason for this lack of understanding is the diversity of protein degradation pathways [7]. Protein aggregation can occur as a result of either chemical or physical instability. Proteins can undergo chemical degradation at individual amino acid residues or at their internal disulphide bonds, and they can also undergo physical degradation by irreversible changes in their secondary or tertiary structure. Ideally, information would be available on the specific environment of each reactive residue. This presents technical challenges to characterise the chemical [7–10] and physical (secondary and tertiary) structure of proteins in the solid glassy state [8,10,11]. A further complexity is that different degradative stresses occur in the multiple processing steps involved in the manufacture of glassy stabilization systems [3], like the stresses that can arise during industrial freeze-drying including vial filling operations, freezing, primary or secondary drying [12,13].

The most commonly proposed mechanisms by which carbohydrate additives stabilize biopharmaceuticals in glasses are based upon two complementary concepts. Firstly, the proposition that

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stabilization is related to the dynamics of the glass state [2,5,14,15], which includes the "vitrification" hypothesis [5,16] and secondly that stabilization is related to the solvent properties of the carbohydrate stabilizing additives, including the "water substitution" hypothesis [17–19].

The "water substitution" hypothesis was based on biomimetic arguments concerning anhydrobiotic organisms which accumulate carbohydrate in response to desiccation stresses [17–19]. The hypothesis is supported by studies using FTIR on protein and protein/carbohydrate mixtures which show the extent to which protein secondary structure is preserved in the dehydrated state and the reversibility of protein secondary structure after dehydration and rehydration [18,20–22]. The disaccharide trehalose was identified for its distinct properties in stabilizing both protein and lipid bilayer structures [19]. However, subsequent experimental studies have shown that sucrose, trehalose and other carbohydrates all possess stabilizing properties with none performing consistently better than the others [2,23,24].

Evidence supporting the role of glass state dynamics has grown progressively. It is relevant to both the processing of biopharmaceutical formulations and their subsequent storage stability. Knowledge of the state diagram of a biopharmaceutical formulation allows a freeze-drying cycle to be developed whilst preventing collapse or matrix crystallisation [3]. Recently, the link between physical and chemical stability and the secondary β- and local high-frequency local relaxations of the formulations has been examined [14,25–27]. Research on antiplasticisation [14,25] and the effects of annealing [26,27] yielded results which potentially have practical utility in enhancing biopharmaceutical product stability.

In this work, we are examining the effect of dilution on the aggregative stability with both dissacharide and polysaccharide in binary globular protein–carbohydrate mixtures. Whereas previous work on the stabilizing effect of polysaccharides have focussed on mixed polymer systems e.g. dextran-polyvinylpyrrolydone [28–30], here we use a pure protein–polysaccharide mixture which has not been studied previously. To make this a comprehensive study of aggregative protein stability in the glass state, the pure protein was included in the experimental design to give data throughout the entire concentration range.

The protein β-lactoglobulin (BLG) was chosen as a readily available well-studied globular protein from the whey of mammalian milk [31]. In food processing, it is commonly vitrified by spray-drying [32] often in the presence of the reducing disaccharide lactose and so there are similarities with biopharmaceutical processing. BLG has a molecular weight of 18.3 kDa and it is resistant to thermal denaturation at low water contents [33]. It occurs predominantly in a dimeric form under physiological conditions [34,35] and the structures of several crystalline polymorphs have been solved [31,36]. On dehydration from dilute solution it has been shown to undergo a colloidal glass transition prior to becoming a true glass at lower water contents [37]. The pure glassy protein undergoes a solid state aggregation reaction with an Arrhenius activation energy of 95 kJ mol^{-1} which can be reduced to negligible rates by the addition of trehalose [38].

The aim of the present study is to examine the role of carbohydrate and carbohydrate content on the glass state stability of the model protein, β-lactoglobulin (BLG). The glass transition behaviour of the protein/carbohydrate mixtures were characterised using differential scanning calorimetry and the aggregation of BLG in the solid state measured by size exclusion chromatography. The effects of the disaccharides, trehalose and sucrose, and the polysaccharide dextran on the rate of the glass state aggregation of BLG were compared and studied. Finally, the mechanistic basis of the results is discussed in relation to the "water substitution" and "vitrification" hypotheses.

2. Materials and methods

2.1. Materials

Sucrose (S7903), D-(+)-Trehalose dihydrate (T9531), β-lactoglobulin (BLG) from bovine milk, mixed isoforms (L3908), monobasic sodium phosphate (S0751) and dibasic sodium phosphate (S7907) were purchased from Sigma–Aldrich. Dextran (31389), MW about 40 kDa, was purchased from Fluka. All compounds were used without further purification.

2.2. Preparation of low water content amorphous protein and its carbohydrate mixtures

Protein and protein/carbohydrate glasses were prepared by freeze-drying 10 wt.% solutions with a range of BLG/carbohydrate ratios made up in 10 mM pH 7.2 phosphate buffer. Two micro litre of each solution were added to Schott clear vials with a capacity of 3.5 mL (fill height 19 mm) and a 13 mm crimp neck and freeze-dried using a Virtis Advantage EL 2.0 freeze-dryer (Biopharma Process Systems, Winchester, UK). The following conditions were used for freeze-drying: freeze the samples until shelf temperature reaches −45 °C; hold at −45 °C for 60 min; hold at −50 °C for 60 min; primary dry at −35 °C for 40 h at 80 mTorr; secondary drying at temperatures rising from −35 °C to −10 °C at 80 mTorr over a period of 54 h (10 h at −32 °C; 20 h at −31 °C, 12 h at −20 °C and 12 h at −10 °C) before increasing the temperature to 20 °C at 80 mTorr for a further 52 h. Samples were then vacuum dried in a vacuum oven at 20 °C over phosphorus pentoxide for 5 days. Finally, the freeze-dried mixtures were closed with a pre-dried rubber stoppers, sealed with aluminium tear-off seal and stored in a freezer until used. The amorphous nature of the samples was confirmed by the absence of crystals when viewed using polarising light microscopy before treating the samples thermally.

2.3. Glass transition temperature and water content determination

Glass transition temperatures (T_g) of freeze-dried samples were determined in triplicate using differential scanning calorimetry (DSC) after weighing 5–10 mg of the dried mixtures into aluminium DSC pans (Perkin Elmer) in a glove box under a dry argon atmosphere. The T_g's were determined using a Perkin Elmer DSC 7 instrument within the temperature interval of 20–120 °C on a re-scan at a scanning rate of 10 °C min^{-1}. The calorimeter was calibrated for temperature with indium and octadecane. Baselines were obtained using an empty pan and all the thermograms were baseline corrected. The T_g was determined as the midpoint of the temperature range over which the change in specific heat occurred [38]. The water content of samples was measured after performing the calorimetry studies by puncturing the pan with a needle and then drying the pan to a constant weight (as determined using a Mettler ME30 balance) in a vacuum oven at 40 °C over phosphorus pentoxide. Samples were cooled to room temperature before weighing.

2.4. Thermal treatments and dissolution

All samples were prepared by weighing 1–2 mg of freeze-dried BLG/carbohydrate mixture into 2 mL vials (Wheaton Shorty vial, Screwneck, Sigma–Aldrich) in a glove box purged with argon. The dried powder was mixed with a spatula to ensure homogeneity of the sample before being transferred to a vial under an argon atmosphere to prevent any water being absorbed. The vials were closed and placed in a thermostatically controlled oven at the desired temperature (70–100 °C) for the required time (24–144 h).

After heating, the vials were cooled in ice-water before dissolving the samples in 2 mL of 10 mM pH 7.2 sodium phosphate buffer to obtain a final sample concentration of 1 mg mL^{-1}. The solutions were mixed horizontally at room temperature with a shaker (Rotatest Shaker, Model R 100/TW, Luckam, speed 5) for 30 min. All samples were prepared and tested in duplicate.

2.5. Size exclusion chromatography

Analysis of aggregation was performed by high-performance size exclusion chromatography (HP-SEC) using a Superose 12HR 10 × 300 mm column (GE Healthcare) with a flow of 0.4 mL min^{-1}. The exclusion limit of the column is 2000 kDa. Prior to running the samples the column was equilibrated in 10 mM pH 7.2 sodium phosphate buffer containing 60 mM NaCl. The sample injection volume was 30 μL. All sample measurements were performed at room temperature. The samples were analysed using a UV detector at 280 nm (Dionex PDA-100 Photodiode Array Detector, deuterium/tungsten). The concentration of BLG and the different species of its aggregates appearing in the elution profile were determined by measurement of their absorbance at 280 nm. The column was calibrated with a gel filtration standard (Biorad) to calculate the apparent molecular weight of the molecules eluted. Peak integrations were performed using Chromeleon software (version 6.8, Dionex). A mass balance on the amount of protein was performed and it was established that full recovery of the protein had been achieved. The initial aggregation rate was determined as the initial slope of the dimer peak area *versus* time plot.

3. Results and discussion

3.1. Characterisation of the state of the freeze-dried BLG/carbohydrate mixtures

In order to study the effect of different carbohydrates on the stability of BLG, different concentrations of protein in the glassy matrix were studied. The formulations examined are described in Table 1. Fig. 1 shows the effect of composition on the calorimetric glass transition temperatures of the BLG/carbohydrate mixtures. All the water contents were below 4.0 wt.% and typically 1.0–2.0 wt.% (see Table 2). For these highly hygroscopic materials, small differences in handling procedures can lead to this variation in water content. The glass transition temperatures of the carbohydrate rich mixtures are of the order expected with deviations from literature values for pure materials due to the small quantities of water and phosphate buffer in the mixtures [24,39–41]. Whereas the BLG/disaccharide mixtures have glass transition temperatures below 100 °C those measured for the BLG/dextran mixtures are in excess of 170 °C as would be expected for polysaccharide based mixtures [42,43].

At BLG contents above 77 wt.%, the calorimetric glass transition of the BLG/carbohydrate mixtures become indistinct and so the transition could not be determined by this method. This is consistent with previous reports in the literature which found that native globular proteins do not exhibit the distinct heat capacity increments at the glass transition [44,45] as observed for disaccharides and polysaccharides [24,39,42,43]. Neglecting the relatively small effects due to the differences in water content, the BLG/sucrose and BLG/trehalose mixtures show an increase in glass transition temperature with increasing BLG content which can be extrapolated to a limiting value of about 95 °C for 100 wt.% BLG. This extrapolation is shown in Fig. 1 and was made using the Gordon–Taylor equation [46] which, for a binary mixture, takes the form:

$$T_{g\ \mathrm{mixture}} = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2} \quad (1)$$

where the glass transition temperature of the binary mixture, $T_{g\ \mathrm{mixture}}$, depends upon the mass fraction of each component w_i, the glass transition of the pure materials, T_{gi} and an empirical constant, k. The extrapolated apparent glass transition temperature of BLG was determined by simultaneously performing a least squares fitting to the BLG-sucrose and the BLG-trehalose $T_g - w_2$ data using the apparent T_g and two k values as adjustable parameters. The constant k was 0.37 and 0.06 for sucrose and trehalose, respectively. Knowledge of the glass transition temperatures of the BLG/carbohydrate mixtures means that, when interpreting the aggregation data, the state of the system is known, that is, whether the system is in the glass state or in the glass transition region at any particular temperature.

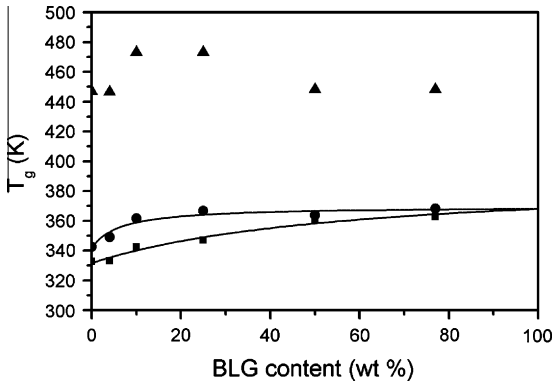

Fig. 1. The effect of BLG content on the calorimetric glass transition temperatures of amorphous freeze-dried BLG/carbohydrate mixtures. Carbohydrate: ■, sucrose; ●, trehalose; ▲, dextran. The dashed lines are fits of the BLG/disaccharide data to the Gordon–Taylor equation.

Table 1
The amounts of protein, carbohydrate and buffer used to prepare the freeze-dried mixtures.

BLG/carbohydrate	BLG (mg)	Carbohydrate (mg)	Buffer (g)
Pure protein	600	0	5.4
91/10	600	60	5.94
77/23	600	180	7.02
50/50	600	600	10.8
25/75	600	1800	21.6
10/90	600	5400	54.0
Pure carbohydrate	0	2400	21.6

3.2. SEC characterisation of thermally induced aggregation of BLG

In order to compare the reaction behaviour of the BLG in mixtures with the different carbohydrates, conditions at which the measurable reaction rates occurred in all systems were sought. Previously thermally induced aggregation in BLG/trehalose mixtures had been shown to occur in the temperature range from 60 to 100 °C over the timescale 48–144 h [38]. In the present study, initial experiments were performed at 100 °C to establish whether similar reactions were occurring in the presence of the different carbohydrate additives and then, subsequently, the majority of measurements were made at 70 °C, a temperature at which firstly, all the systems in which aggregation was observed were glassy, and also, at which measurable aggregation was observed over experimental timescales. The 9 wt.% BLG/sucrose mixture with a glass transition temperature of 69.2 °C was in the glass transition region at 70 °C but no measurable aggregation was observed in this sample. During the thermal challenge, no visible change was ob-

Table 2
The glass transition temperatures and residual water contents of the freeze-dried BLG/carbohydrate mixtures.

BLG/carbohydrate (w/w)	Trehalose		Sucrose		Dextran	
	T_g (°C)	Water content (wt.%)	T_g (°C)	Water content (wt.%)	T_g (°C)	Water content (wt.%)
Pure carbohydrate	69.4	3.9	59.4	0.35	173.6	1.4
4/96	76.0	0.8	59.9	0.54	173.3	0.5
10/90	88.5	0.1	69.2	0.14	>200	0.9
25/75	93.5	2.1	73.8	0.60	>200	0.3
50/50	90.6	2.1	87.5	1.88	>175	0.2
77/23	>95	0.4	89.4	1.25	>175	0.4
91/9	–	2.5	×	1.29	>175	1.3
Pure BLG	×	1.5	×	1.5	×	1.5

served, so it was assumed that the structure and appearance of the samples was preserved, which is consistent with the samples remaining in an amorphous state without buffer recrystallisation or structural collapse [3,9,40]. In our previous studies of lysozyme/carbohydrate mixtures extensive carbohydrate hydrolysis and protein glycation only occurred in those systems which showed discolouration and structural collapse [9]. Only relatively minor chemical modifications were observed in those systems which preserved their physical appearance.

Prior to characterisation using size exclusion chromatography, samples were reconstituted in buffer after having been subjected to a thermal challenge. As described previously [38], this procedure was thoroughly checked to establish that the SEC elution profile was independent of any of the variations in the reconstitution and analysis procedure e.g. dissolution time, dissolution conditions, waiting time in HPLC carousel prior to injection. Fig. 2 shows the chromatograph of a 77 wt.% BLG/dextran sample which was held at 100 °C for 48 h. Detecting at a wavelength of 280 nm means that only the protein is observed and so absorbance is solely due to the BLG and its aggregates. The profile of all the chromatographs followed a similar pattern. The large peak with a peak retention time of about 32 min corresponds to the unreacted BLG which elutes as a dimer under these conditions [34]. The second peak elutes at 29.3 min. In practise, the retention times showed small shifts depending upon composition, in particular for the dextran mixtures, probably because the BLG and dextran coelute though, as dextran does not absorb at 280 nm, it is not apparent in the chromatograph. The column was calibrated using a set of molecular weight (MW) markers. A regression of log (MW) *versus* elution time yielded a linear calibration curve for the column. This allows estimation of the molecular weight of the species eluted in the peaks from the peak retention times which yields MW_1 = 31.0 kDa, MW_2 = 60.4 kDa and the ratio MW_2/MW_1 = 1.95 where the subscript refers to the peak numbering in Fig. 2. Thus, although the absolute estimate of the dimer molecular weight is slightly low (literature MW for dimer = 36.6 kDa [31,36]), by taking ratios the second peak can be confidently assigned to a tetramer peak (and peak 3 to a hexamer peak) as shown previously [38].

The qualitative effect of increasing the carbohydrate content to the BLG/carbohydrate mixture can be seen by directly comparing chromatograms. Fig. 3 shows chromatograms of samples of BLG-carbohydrate mixtures which were held at 70 °C for 96 h. The absorbance of each elution profile has been scaled so as to have the same total integrated area as the pure BLG control sample. Furthermore, for purposes of comparison, the peak retention times have been brought into coincidence by scaling time with the appropriate elution times for the void volume, t_v, and excluded volume, t_e. The elution profiles in Fig. 3 show that the BLG control

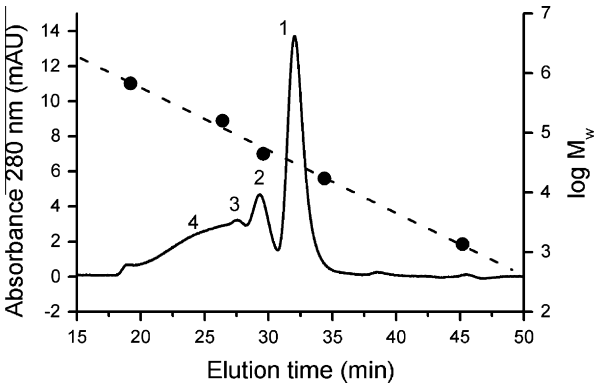

Fig. 2. SEC elution profile of BLG-dextran mixture containing 77 wt.% BLG heated at 100 °C for 48 h and elution times of molecular weight markers. The dashed line is a linear regression to the log M_w – elution time data. Calibration indicates that the elution peaks correspond to dimers (1), tetramers (2), hexamers (3) and larger aggregates (4).

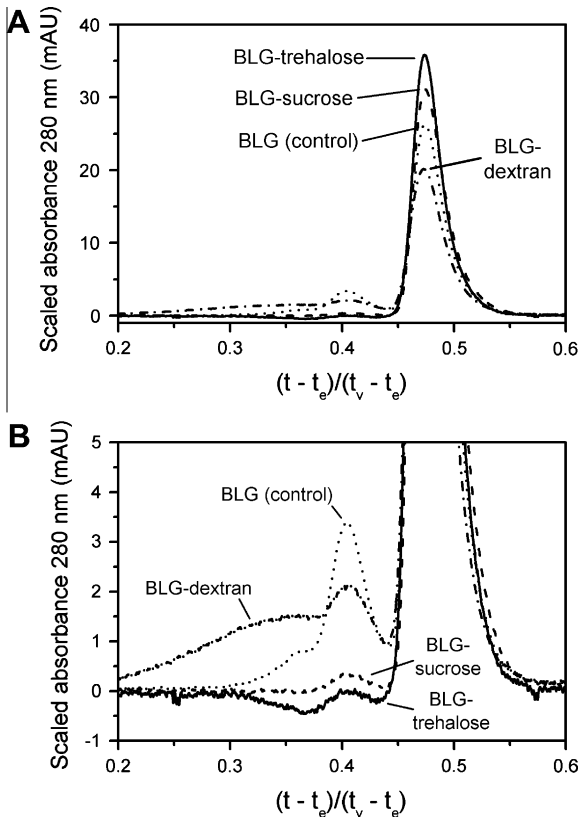

Fig. 3. SEC chromatograms showing the effect of carbohydrate on the aggregation of BLG in 25 wt.% BLG/carbohydrate glassy mixtures after 96 h at 70 °C. Full chromatogram (A) and expanded view of the same chromatogram (B) showing the tetramer peak in the centre (e.g. labelled BLG (control)), the base of the dimer peak on the right and the absorbance arising from the larger aggregates with shorter elution times on the left which occurred in BLG-dextran samples.

sample has the highest tetramer peak though it still contains more dimers than the BLG/dextran sample. The two BLG/disaccharide samples show least reaction with very few tetramers in either sample. The most extensive reaction is exhibited by the BLG/dextran sample which shows a broad range of aggregate sizes. Our detailed study of the BLG/trehalose system [38] established that rates of aggregation can be quantified through the variation of the integrated peak areas as reported in the next section.

3.3. The effect of carbohydrate addition on aggregation kinetics

A quantitative measure of aggregation rate can be obtained from the variation of the integrated peak areas with time by measuring the disappearance of dimers. Fig. 4 shows the variations of the dimer peak area (peak (1) in Fig. 2) with time for the three BLG/carbohydrate mixtures at four carbohydrate contents. For the purposes of comparison, the pure BLG control is also included in each figure. The aggregation behaviour varies with each variable (time, carbohydrate and carbohydrate content) with the exception of the change between the two disaccharides, sucrose and trehalose. The behaviour of the two disaccharides is similar at all carbohydrate contents. With increasing disaccharide from 9 to 50 wt.% there is a reduction in the rate at which the dimer peak area decreases with time. Whereas at 50 wt.% disaccharide the rate of aggregation has fallen to low levels, at 75 wt.% disaccharide no significant aggregation is detected. At short time ($\leqslant$48 h) and relatively low dextran content (9 wt.% and 23 wt.%), the rate of reduction of the dimer peak area is similar to that of the BLG-disaccharide mixtures. However, at longer times (96 h) and at all dextran contents, there is a pronounced reduction of the dimer peak area such that it falls below that observed for the BLG control. In terms of stabilization with respect to aggregation, the addition of dextran has made the BLG less stable.

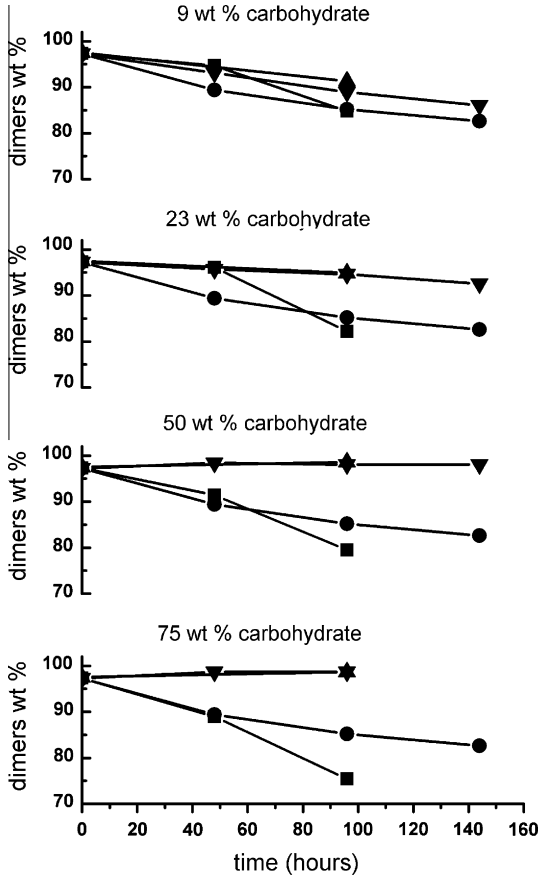

Fig. 4. The effect of carbohydrate, carbohydrate content and heating time on the BLG dimer elution peak area after heating the BLG/carbohydrate mixtures at 70 °C. Compositions: ●, BLG (control); ▲, BLG/sucrose; ▼,BLG/trehalose; ■, BLG/dextran.

Initial rates of BLG dimer disappearance, k_2, were calculated from the initial gradients of the curves in Fig. 4. In order to obtain a comparative measure of the effect of the addition of the carbohydrate, these rates were normalised by dividing by the rate of dimer disappearance for pure BLG, k_2^0, and these ratios are shown in Fig. 5. This clearly summarises and quantifies the short time behaviour shown in Fig. 4. At low carbohydrate contents, the effect of dextran is comparable with that of sucrose and trehalose. However, once the carbohydrate content exceeds 23 wt.%, while the rate of aggregation for the BLG-disaccharide mixtures falls to negligible values, the rate in the presence of dextran increases until it exceeds the rate in the pure BLG control. At the highest carbohydrate concentration (96.0 wt.%), the rate decreases to negligible values for all the carbohydrates.

The aggregation behaviour of the BLG in the BLG/dextran mixtures at concentrations above 25 wt.% changes qualitatively. Whereas at the lower concentrations the main aggregation product observed is the tetramer, at the 50 wt.% and 75 wt.% dextran concentrations the aggregation process has progressed further producing significant quantities of larger aggregates as shown in Fig. 6A. At 90 and 98 wt.% dextran, the extent of aggregation reduces to low values as shown in both Fig. 6A and Fig 5. These subtle concentration effects disappear at higher temperatures as shown in Fig. 6B for the BLG/dextran mixtures held at 100 °C for 48 h. Very extensive aggregation is observed throughout the concentration range, with the lowest rate of aggregation occurring at 50 wt.% dextran, almost the exact opposite of what was occurring at 70 °C. This suggests that additional aggregation mechanisms may be occurring at 100 °C. The extrapolated glass temperature of the pure BLG (Fig. 1) is 95 °C and so at 100 °C this material is slightly above its glass transition. However, the calorimetric glass transitions detected in the BLG/dextran mixtures in concentrations of dextran above 25 wt.% are in the range 175–200 °C and so at 100 °C the mixtures are well below this temperature which indicates that, for these formulations, at least a proportion of the material is in the glassy state.

3.4. The origin of the concentration dependence of thermally induced BLG aggregation in the glass state

For BLG/trehalose mixtures, we have proposed that dilution is a main factor controlling the effect of the addition of carbohydrates

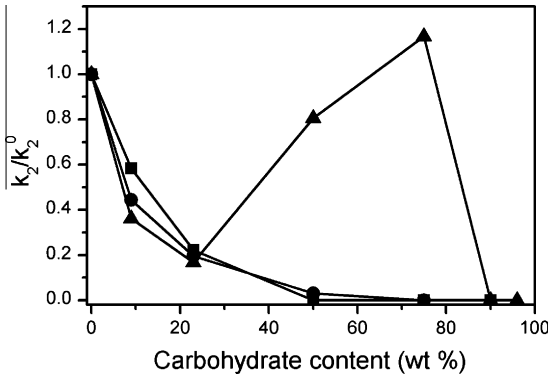

Fig. 5. The effect of carbohydrate and carbohydrate content on the rate of disappearance of BLG dimers for BLG/carbohydrate mixtures heated at 70 °C. The rate is expressed as a ratio with the rate of BLG dimer disappearance for pure BLG, k_2^0. Compositions: ■, BLG/sucrose; ●, BLG/trehalose; ▲, BLG/dextran.

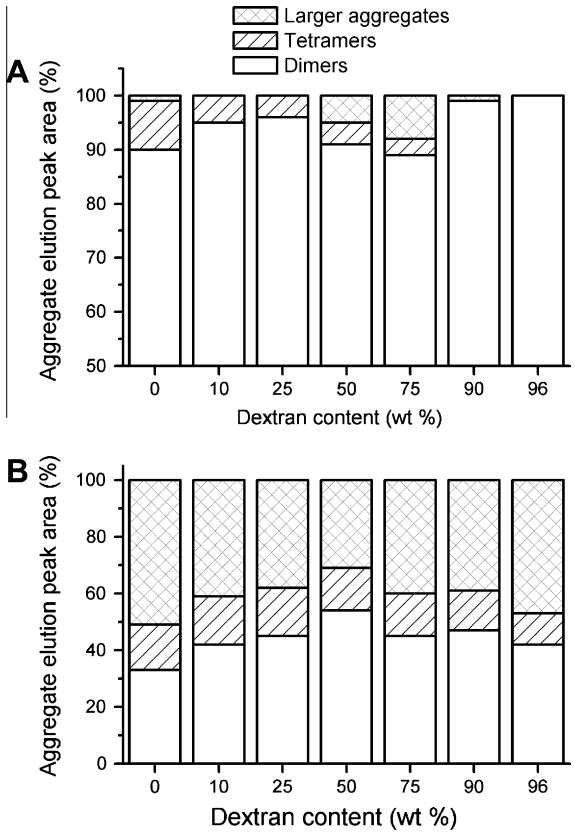

Fig. 6. Histograms showing the BLG aggregate distribution after low water content amorphous BLG/dextran mixtures have been held at (A) 70 °C and (B) 100 °C for 48 h.

in the aggregation of BLG in the glassy state [38]. The experimental data show a decrease in aggregation rate in the mixtures with the progressive reduction in BLG concentration as carbohydrate is added starting from 100 wt.% BLG and decreasing to 4 wt.%. Dilution is the straightforward explanation of this effect. Other explanation such as the vitrification hypothesis does not concern the concentration effect, and the water replacement hypothesis as originally stated, invoked specific interactions particularly of the disaccharide trehalose. The behaviour of BLG/sucrose mixture is similar to that of BLG/trehalose and so it is likely the same mechanisms occurs. Furthermore, BLG/dextran shows the similar short time behaviour at 9 and 23 wt.% carbohydrate and so at these concentrations the same explanation would apply. The current results show that, at low concentrations, trehalose, sucrose and dextran exhibit similar behaviour and so there is no evidence for specific interactions at these concentrations. These data are consistent with that of Yoshioka et al. [2] and Wang et al. [6] who found that rates of aggregation show an exponential decrease with the amount of carbohydrate added.

However, when the polysaccharide dextran is added at concentrations in excess of 23 wt.% the aggregation behaviour increases to rates which are similar to those found in the pure protein and dilution no longer explains the observed concentration dependence. This could be due to a phase separation of the mixture into BLG rich and dextran rich phases during the preparation of the glasses as observed for other protein/polymer mixtures by Izutsu and Kojima [47]. Prior to freeze-drying the BLG/dextran mixtures (10 wt.%) were transparent single phase solutions, however, during freezing and subsequent drying, the BLG and dextran concentrations increase, firstly due to freeze-concentration and then, subsequently, due to further water loss during the secondary drying stage [3]. In their analysis of the miscibility of BLG and dextran mixtures, Schaink and Smit reported that the position of the phase boundary depends upon pH, ionic strength and the molecular weight of the dextran [48]. They showed that BLG and dextran become partially miscible at a total polymer (BLG + dextran) concentration of about 12 wt.% for 50/50 BLG/dextran mixtures at ambient temperatures. This suggests that globular protein–polymer immiscibility during freeze-drying could be a precursor to loss of stability.

4. Conclusions

The role of carbohydrates in glassy protein–carbohydrate preservation systems has been examined. It was found that mixtures containing BLG and the disaccharides sucrose or trehalose show similar protein aggregation behaviour that could be explained by a diluent effect of the glass forming carbohydrate. The aggregation rate falls monotonically from a maximum rate for the pure glassy protein to a negligible rate at 75 wt.% carbohydrate. However, when the polysaccharide dextran is used, BLG/dextran mixtures only followed this behaviour over a limited range of compositions. Based on the known phase behaviour of BLG mixtures at ambient conditions [48], it is proposed that the deviation in aggregation behaviour may be due to phase separation. The extent of phase separation would determine the composition and proportion of protein rich and polysaccharide rich phases, in a highly heterogeneous structure. This suggests that the criterion for choosing a stabilizing carbohydrate is that it should be miscible with the protein at all compositions and temperatures along the processing trajectory. In a general way, this supports both the water substitute and the vitrification hypotheses [1,5,17] and the view that "glass formation is necessary but not sufficient for stabilization of proteins during freeze-drying" [49].

Acknowledgements

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