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Effect of saccharides on glass transition temperatures of frozen and freeze dried bovine plasma protein

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ABSTRACT

In this study, to preserve the integrity of plasma protein, protective agents, such as saccharides are added to produce a glassy (vitrified) state. Differential scanning calorimetry (DSC) was used to measure the glass transition (T_g), crystallization temperatures (T_c) of the solid freeze–dried bovine plasma protein and the glass transition temperature (T'_g) of the protein freeze solution, with the addition of inulin as protective agent, comparing the behavior with glucose and sucrose. The results indicated that transition temperatures increased with the molecular weight of the saccharide, conferring inulin a stabilizing effect at higher storage temperature. The T'_g and the water plasticizing effect were estimated by means of two theoretical models: Miller/Fox and Gordon/Taylor extended for multi-component systems. The determination of the glass transition temperatures is useful in defining a freeze–drying cycle and storage stability of plasma protein concentrates.

1. Introduction

The use of inulin and its derivatives in the food industry is in constant increase mainly by their technological and nutritional benefits. These benefits are, fat and sugar replacement, low caloric bulking agent, texturing and water-binding agent and it has a prebiotic activity (Kip et al., 2006; Ronkart et al., 2009). Commercial inulin is mainly extracted from chicory root and is available as a spray-dried powder product. In a previous work, we investigated the inulin capacity as protective agent of proteins during freeze–drying process, and it was demonstrated that the denatured proteins percentage was reduced preserving their conformation, and consequently, their functional properties, even during storage (Rodriguez Furlán et al., 2010).

Freeze–drying is the main process used to produce stable proteins, which are unstable in aqueous solution with limited shelf life. An appropriate choice of stabilizers (saccharides) is needed to protect the proteins from denaturation during processing, as well as to provide a glassy matrix required for long-term storage stability in the dried solid (Costantino et al., 1998; Liao et al., 2004; Passot et al., 2005). Primary drying is the most time consuming stage of the process. It should be carried out at the maximum allowable temperature usually associated to the glass transition temperature of the maximally freeze concentrated solution (T'_g). Below this temperature a glassy state that behaves as an amorphous solid is obtained. If the temperature of the frozen system rises above the T'_g, the material becomes less viscous and freeze–drying may cause the loss of the porous structure and product collapse (Chen and Oakley, 1995; Passot et al., 2005).

In the freeze–dried sample, water is removed and the solute concentration in the matrix increases, obtaining a material with an amorphous structure that exhibits a glass transition temperature (T_g) (Chen and Oakley, 1995; Shah and Schall, 2006). The T_g can be defined as a second order phase change temperature at which sample properties change from glassy state to rubbery state (Gallegos Infante et al., 2005; Noel et al., 1995; Roos, 1995). It is also defined in kinetic terms as the temperature below which the viscosity of a liquid is at least 10^{13}–10^{14} Pa s (Chen and Oakley, 1995; Katkov et al., 2006). Besides, it has been assumed that amorphous products are stable in their solid, glassy state below T_g with a high internal viscosity. As the temperature is increased above T_g, various properties of the materials may change, like an increase in the molecular mobility and a decrease in the viscosity, often resulting in a crystallization event of the added solute increasing also food deterioration (Gallegos Infante et al., 2005; Roos, 1995; Shah and Schall, 2006). Therefore, the T_g determines the product stability during storage (Katkov and Levine, 2004).

Both transitions T'_g and T_g are important parameters in the development of the freeze–drying cycle because not only ensures

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product stability and quality, but also allow to improve the efficiency of the manufacturing process (Chen and Oakley, 1995; Passot et al., 2005; Shah and Schall, 2006; Tattini et al., 2005).

Differential scanning calorimetry (DSC) is a tool used to characterize the freeze–drying behavior of protein formulations. There are limited data for the glass transition temperatures of multi-component mixtures and few studies comparing experimental and predicted values of T_g for such mixtures (Shah and Schall, 2006).

The objectives of the present work were: to apply DSC analysis to assess the effect of different saccharides (glucose, sucrose and inulin) on several transition temperatures: (a) the glass transition temperature of the maximally concentrated frozen solutions (T_g') of bovine plasma protein and to compare the experimental results with the predictive equations of Miller/Fox and Gordon/Taylor extended for multi-component systems; (b) the glass transition (T_g) of the freeze dried multi-component mixtures with the objective of improving the lyophilization cycle; (c) the onset crystallization

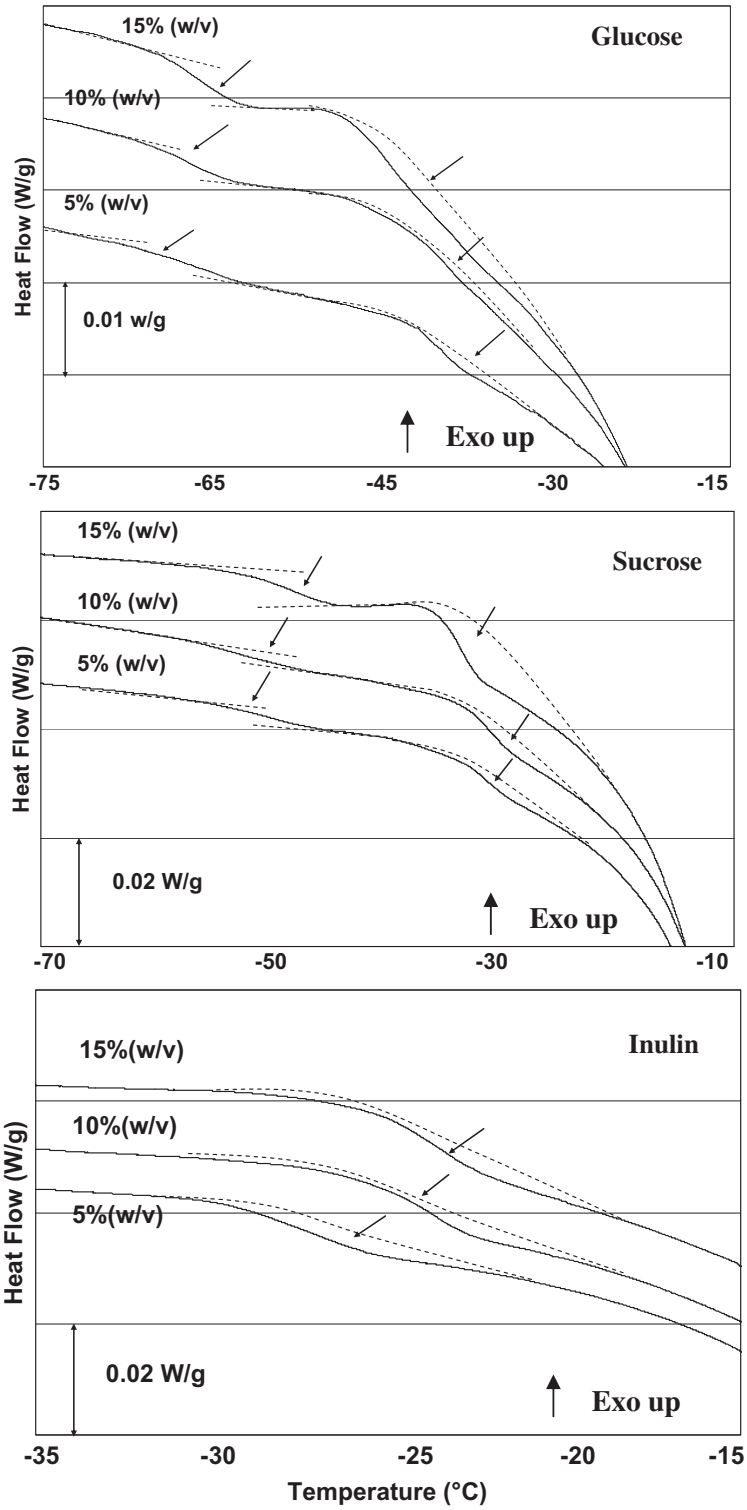

Fig. 1. DSC thermograms for freeze bovine plasma protein–saccharide solutions. Down-arrows indicate T_g'.

Table 1
Effect of type and concentration of cryoprotectant on glass transition temperature (T'_g) of freeze bovine plasma proteins solutions (heating rate: 2 °C/min).

Saccharide	Concentration (w/v) (%)	T'_{g1} (°C)	T'_{g2} (°C)
Glucose	5	−62.50 ± 0.58[a]	−39.24 ± 0.75[a]
	10	−61.06 ± 0.45[a,b]	−39.91 ± 0.83[a]
	15	−59.82 ± 0.68[b]	−44.96 ± 0.49[b]
Sucrose	5	−51.48 ± 1.05[c]	−31.15 ± 0.40[c]
	10	−50.12 ± 1.03[c,d]	−31.86 ± 0.60[c]
	15	−48.42 ± 0.98[d]	−33.72 ± 0.45[d]
Inulin	5	−26.96 ± 0.68[e]	–
	10	−23.67 ± 0.55[f]	–
	15	−22.40 ± 0.45[f]	–

Values represents the means ± standard deviation; $n = 3$. Values followed by different letters in the same column are significantly different from each other ($p < 0.05$).

temperature (T_c) of the solute at temperatures above T_g, in the freeze dried samples.

2. Materials and methods

2.1. Raw materials

Spray dried bovine plasma (Yerubá S.A. Argentina) has been used. The molecular weights of proteins are in the range of 15,000–80,000 Da. The composition is 76 ± 5% proteins, <0.1% fat, 10% ash, 4% water, and 1% low molecular weight compounds.

The saccharides used as protective agents were: commercial sucrose (Ledesma S.A., Argentina) with a purity of 99.99%, glucose (Parafarn, Argentina) and inulin provided by Orafti Chile S.A., obtained from chicory. The commercial inulin employed is mainly constituted by linear chains of fructose, with a glucose terminal unit, and has a molecular weight of 2400 g/mol.

2.2. Preparation of protein/saccharide mixtures: concentration of bovine plasma proteins through ultrafiltration and freeze–drying

It was demonstrated previously that the treatment of bovine plasma proteins by membrane technology: microfiltration, ultrafiltration and diafiltration (MF–UF–DD) allowed protein concentration, eliminating insoluble macroscopic components and reducing the saline content (Rodriguez Furlán et al., 2010). Therefore the same procedure was applied. Bovine plasma at 3% w/v was used as feed solution. A porous support (Viledon F 02431 D, Germany) and a membrane of microfiltration (pore size of 60 μm, Gora, Argentina) were used to remove macroscopic aggregates protecting the UF membrane from fouling. The UF was performed using a Pellicon cassette module (Millipore, Bedford, MA, USA), containing modified polyethersulfone membranes with a molecular weight cut-off of 10-kDa. The discontinuous diafiltration (DD) process, the feed solution was the UF concentrate, which was diluted to the initial volume (3–5 L) with treated water in a single state and ultrafiltrated to the desired concentration. The bovine plasma protein concentrate (concentration: 4% w/v) was fractioned: (a) one fraction considered as witness sample was reserved and (b) the protective agent (glucose, sucrose or inulin) was added to the rest, in concentrations of 5%, 10% and 15% (w/v). A part of these solutions was reserved for DSC analysis to determine T'_g and the others were placed on stainless steel trays, frozen in a freezer at −40 °C and freeze–dried using a lyophilizer (Rificor S.A., Argentina) at 1 bar for 48 h. The sample temperature was measured by a temperature sensor.

Table 3
Data from references used in the calculation of T'_g by Miller/Fox and Gordon/Taylor modified equation.

Data		T'_g (°C)	T_g (°C)	Reference
Water			−135	Georget et al. (1999)
Plasma protein		−11 ± 2		Chen and Oakley (1995) (T'_g)
Glucose	5% (p/v)	−85		Berliner and Reuben (1989)
	10% (p/v)	−79		
	15% (p/v)	−72		
Sucrose	5% (p/v)	−59		Fennema (1996)
	10% (p/v)	−53		
	15% (p/v)	−46		
Inulin	5% (p/v)	−17		Hinrichs et al. (2001)
	10% (p/v)	−15		
	15% (p/v)	−13		

Table 4
Effect of type and concentration of cryoprotectant on glass transition temperature (T_g), crystallization temperature (T_c) and crystallization enthalpy (ΔH_c) of freeze–dried bovine plasma (heating rate: 2 °C/min).

Saccharide	Concentration (w/v) (%)	T_g (°C)	T_c (°C)	ΔH_c (J/g)
Glucose	5	16.31 ± 0.38[a]	73.93 ± 1.40[a]	0.14 ± 0.03[a]
	10	41.52 ± 0.29[b]	78.57 ± 1.58[b]	1.65 ± 0.04[a,b]
	15	60.31 ± 0.48[c]	75.70 ± 1.69[a,b]	0.61 ± 0.02[a,b]
Sucrose	5	48.01 ± 0.56[d]	74.33 ± 1.39[a,b]	2.29 ± 0.09[b]
	10	52.48 ± 0.52[e]	86.15 ± 1.82[c]	15.84 ± 1.2[c]
	15	64.28 ± 0.46[f]	77.01 ± 1.65[a,b]	1.99 ± 0.08[b]
Inulin	5	48.85 ± 0.35[d]	85.27 ± 1.42[c]	0.84 ± 0.03[a,b]
	10	66.18 ± 0.69[g]	102.54 ± 1.78[d]	7.26 ± 0.59[d]
	15	69.25 ± 0.45[h]	79.33 ± 1.57[b]	5.23 ± 0.87[e]

Values represents the means ± standard deviation; $n = 3$. Values followed by different letters in the same column are significantly different from each other ($p < 0.05$).

Table 2
Glass transition parameters for plasma bovine proteins and saccharide solutions.

Saccharide (w/v)		ρ (g cm^{-3})*	T'_g (°C) (Miller/Fox)	Difference (°C)	T'_g (°C) (Gordon/Taylor modified)	Difference (°C)	k
Glucose	5%	1.039	−63.99	1.49	−62.69	0.19	3.5
	10%	1.042	−60.46	0.60	−61.26	0.20	
	15%	1.059	−56.1	3.72	−60.03	0.21	
Sucrose	5%	1.033	−54.07	2.59	−51.38	0.10	4.1
	10%	1.041	−51.24	1.12	−50.58	0.46	
	15%	1.056	−47.04	1.38	−48.47	0.05	
Inulin	5%	1.032	−30.43	3.47	−26.70	0.26	4.5
	10%	1.039	−23.64	0.03	−24.23	0.56	
	15%	1.049	−19.51	2.89	−22.11	0.29	

* ρ Solution density ($T = 19.8$ °C).

2.3. DSC measurements

The solutions containing plasma proteins–saccharides mixture were analyzed to determine T'_g; the freeze–dried solids were monitored to measure T_g and T_c. The DSC determinations were carried out by a Q100DTA Instrument (USA) using liquid nitrogen. Samples (approximately 10 mg) were weighed into aluminum DSC pans, hermetically sealed, and then loaded onto the DSC instrument at room temperature, using an empty pan as a reference.

Solutions were: (a) equilibrated at 20 °C and held for 1 min; (b) cooled at 2 °C/min until −80 °C for glucose, −60 °C for sucrose and −40 °C for inulin and held for 30 min; (c) warmed up to the annealing temperature (−50, −40 and −20 °C, for glucose, sucrose and inulin, respectively) by employing an annealing time of 30 min at heating rate of 2 °C/min (Sunooj et al., 2009); (e) recooled at the same temperature of step (b) and held for 30 min; (f) warmed up to 0 °C at heating rate of 2 °C/min. The effectiveness of the procedure was verified corroborating the absence of ice devitrification in thermograms, that is to say the nonexistence of an exothermic peak previous to the ice fusion.

Freeze–dried solids were equilibrated at 0 °C, held for 1 min and then warmed up to 200 °C at heating rate of 2 °C/min. Measurements were carried out on three separate samples (replicates).The T'_g and T_g were determined from the midpoint of the transition of the baseline shift on the amorphous sample.

In the freeze dried samples, at temperatures above T_g, the onset crystallization temperature (T_c) of the added solute was determined from the intersection of the baseline and the tangent of the exothermic peak. The enthalpy change involved in the overall heat-induced reactions within the protein molecules, ΔH_c, was determined by integrating the area beneath the exothermic peak and above a straight baseline drawn between the beginning and end of the transition temperature range (Akköse and Aktas, 2008; Cao et al., 2008; Chen and Oakley, 1995).

2.4. Theoretical equations for T'_g prediction

For the determination of T'_g dependence with the composition in a multi-component system it is possible to use the Miller/Fox equation, assuming the variation of density of solutions with temperature to be constant (Fox, 1956; Miller et al., 1997; Shah and Schall, 2006). For a ternary mixture, it can be written as:

$$\frac{1}{T_g} = \frac{m_1}{m_t T_{g1}(\rho_1/\rho_t)} + \frac{m_2}{m_t T_{g2}(\rho_2/\rho_t)} + \frac{m_3}{m_t T_{g3}(\rho_3/\rho_t)} \quad (1)$$

where T_g, glass transition temperature; m, mass; ρ, density; the subscripts t, 1, 2, 3 mean total, and each pure component, respectively.

The Gordon and Taylor equation (1952) predicts the plasticizing effect of water on the T_g for a multicomponent system. The equation has been used among others, for systems treated as binary mixtures, determining experimentally the glass transition of the respective solid (Georget et al., 1999; Maitani et al., 2008). Instead we propose a system considering each individual component: bovine protein concentrate, saccharide and water, with each corresponding property:

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2} + k^2 w_3 T_{g3}}{w_1 + k w_2 + k^2 w_3} \quad (2)$$

where w_1, w_2, w_3, are the weight fraction of each component defined as (m_i/m_t), and k is an empirical constant proportional to the plasticizing effect of water. This parameter was calculated to fit experimental data from a nonlinear optimization procedure (Gauss Newton procedure) using software Excel 2003 (Microsoft).

Eqs. (1) and (2) were used for the determination of T'_g of the frozen solutions.

3. Results and discussion

3.1. Effect of saccharides on glass transition of the freeze concentrated matrix

For better preservation of frozen or freeze–dried foods, the storage temperature should be at a temperature below the glass transition temperature as was previously established (Chen and Oakley, 1995; Maitani et al., 2008; Roos, 1995; Salman et al., 2006).

Thermograms of bovine plasma solutions with different saccharides, obtained in a single scan, are shown in Fig. 1. The traces revealed the existence of two glass transitions (T'_{g1} and T'_{g2}) for glucose and sucrose as protective agent, evident as deviations in the base line. Similar results were found by Telis and Sobral (2002) who worked with freeze–dried tomato. This phenomenon can be attributed to separate phases formed by sugars and water and natural macromolecules (proteins) present in the frozen solution (Ohkuma et al., 2008; Sun et al., 1998; Telis and Sobral, 2002; Verghoogt et al., 1994). The transition events reflected that T'_{g1} and T'_{g2} increased and decreased respectively with increasing sugar content. However a constant average value was maintained between both T'_g for each sugar, being −51.2 ± 0.8 and −41.1 ± 0.1

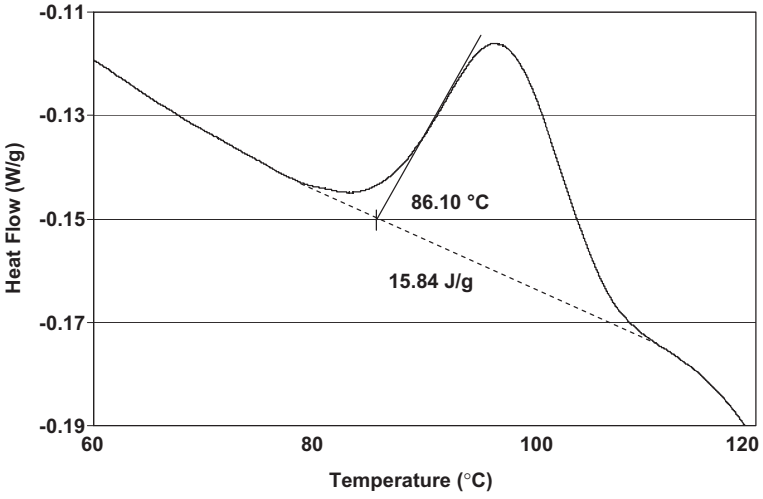

Fig. 2. DSC thermogram for freeze–dried bovine plasma protein with sucrose at 10%. The exothermic event indicates T_c.

for glucose and sucrose, respectively. Similar results were found by Ohkuma et al. (2008) on freeze–dried surimi product with trehalose.

For inulin an only T'_g was found, which increased with the increase of saccharide concentration. Furthermore, the results showed that in the case of freeze solutions for the same saccharide concentration, T'_g (Table 1) increased with the molecular weight of the cryoprotectant that is: inulin > sucrose > glucose. This behavior suggests that inulin has a greater cryostabilizing effect on bovine plasma proteins than other sugars, improving product stability. The same tendency was reported previously by means of the evaluation of protein denaturation, functional properties, and shelf life time (Rodriguez Furlán et al., 2010). By the other hand, Hinrichs et al. (2005) found that inulin exhibit better stabilizing properties than sucrose and trehalose in the prevention of the nonPEGlated lipoplexes aggregation. Besides, Ohkuma et al. (2008) established that T'_g of freeze–dried surimi depended strongly on the types and content of sugar and that at each sugar level the T'_g was trehalose > sucrose > glucose > sorbitol and also many other studies concluded that transition temperatures increased with the saccharide molecular weight (Chen and Oakley, 1995; Roos, 1995; Schenz,1995).

These results indicated that for freeze–drying in which the processing conditions and costs are linked directly to T'_g of the frozen solution, it is important to note that the higher value of T'_g as in frozen solutions with inulin, allow higher freezing temperatures during processing and so greatly reducing production costs.

Water plasticizers amorphous food materials and so decreases drastically T'_g of food polymers (Noel et al., 1995). The equations of Miller/Fox and Gordon–Taylor were used to predict the effect of water on T'_g and compared with experimental values (T'_g from Table 1) for a multicomponent system formed by proteins, saccharides and water (Table 2). The data of T'_g of all pure components required for the Eq. (1) are listed in Table 3. The densities of bovine plasma proteins, glucose, sucrose and inulin (at room temperature) were determined with a digital densimeter, and the results were: 0.4 ± 0.08 g/cm^3, 0.6 ± 0.05 g/cm^3, 0.8 ± 0.04 g/cm^3 and 0.3 ± 0.05 g/cm^3, respectively. The T_g value of bovine plasma protein for Eq. (3), was 65 ± 3 °C. Entering this data into Eqs. (1) and (3), the predicted values of T'_g were obtained, they are listed in Table 4. The results showed that the glass transition property evaluated from the proposed models was in agreement with the experimental data with an average error of 4.86% for the Miller/Fox equation and 0.09% for Gordon/Taylor equation.

The value of k from the Gordon/Taylor equation corresponds to resistance to a T'_g decrease induced by the plasticizing effect of water (Georget et al., 1999; Noel et al., 1995; Ohkuma et al., 2008). The order found for k value of the saccharides was: inulin > sucrose > glucose. Despite the higher plasticizing effect for inulin, this sample had the highest T_g values therefore a higher product stability, as has been previously established.

3.2. Effect of saccharides on glass transition of the lyophilized samples

The glass transition is often followed by crystallization of the solutes (exothermic peak) where the molecular mobility increases and the sample crystallizes into the thermodynamically stable state (Roos, 1995; Shah and Schall, 2006; Tattini et al., 2005). As an example, an exothermic peak due to sucrose crystallization can be observed in the Fig. 2. Crystallization causes the most drastic changes on physical properties of food polymers and it affects considerably food stability. The crystallization temperature (T_c) obtained from the intersection of the baseline and the tangent of the exothermic peak, and the crystallization enthalpy (ΔH_c) for the different protective agents at different concentrations, are listed in Table 4. The tendency to crystallize depends on size and concentration of stabilizers (Roos, 1995; Tattini et al., 2005). The results showed that in the case of freeze–dried solutions for the same saccharide concentration T_c increased with the molecular weight of the cryoprotectant. Table 4 show an increase of ΔH_c when the saccharide concentration was 10% (w/v), indicating a higher amorphous content. It can be due to a suitable proportion of sugar and protein in the mixture that allows a better interaction among these components (Dilworth et al., 2004; Gabbott, 2008).

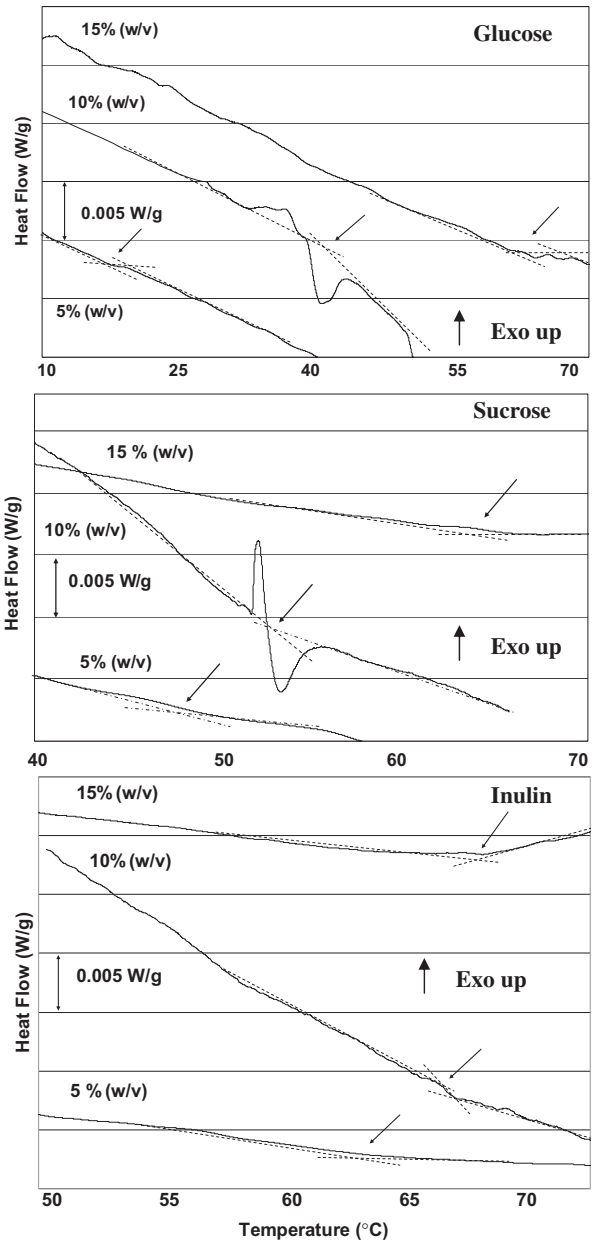

Fig. 3. DSC thermograms for freeze–dried bovine plasma protein–saccharide mixtures. Down-arrows indicate T_g.

The thermogram of Fig. 3 shows T_g of freeze–dried samples with variable content of glucose, sucrose and inulin. The results are listed in Table 4. As expected, freeze–dried plasma protein/saccharide mixtures were in the glassy state and it is shown that T_g of the sample increases with increasing saccharide concentration. Similar results were found by Shah and Schall (2006), and by Tattini et al. (2005). This effect can be explained considering that sugar forms hydrogen–bridge bonds with proteins reducing available volume for the interaction with water molecules, so water become less

effective as plasticizer with an increase in sugar content (Gabbott, 2008).

Processes of devitrification and hence product spoilage can occur if the temperature of storage is higher than the T_g of the sample. Therefore, a higher T_g provides greater stability at higher temperatures, reducing the storage costs.

4. Conclusions

The glass transition properties of solution and freeze–dried of bovine plasma proteins–saccharides mixtures were investigated in this study. It was demonstrated that the bovine plasma proteins–inulin mixtures have the highest glass transition temperature for the protein solution as for the freeze–dried powder, optimizing the freeze–drying process and also the storage conditions below the collapse temperature of the material. Thermograms revealed the existence of two glass transitions in solutions (T'_{g1} and T'_{g2}) for glucose and sucrose. With increasing sugar content, the T'_{g1} and T'_{g2} of the samples increased and decreased, respectively. For inulin only one T'_g was found, which increased with saccharide concentration. T'_g, T_g and T_c depended on the molecular weight of saccharides, increasing with the increasing of molecular weight, being inulin > sucrose > glucose.

The proposed model allowed the prediction of transition temperature in a multicomponent mixture which is useful to design a freeze–drying cycle and storage stability of plasma protein concentrates.

Therefore, the results showed biggest values of T_g in the freeze–dried samples of inulin proven a better stabilization properties of plasma protein during storage than mono and disaccharides such as glucose and sucrose preventing the unfolding of bovine plasma proteins to higher temperatures. Furthermore, the higher T'_g of freeze solutions of bovine proteins with inulin allows higher freezer temperatures during freeze–drying, reducing the production cost.

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