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Calorimetric Study of Inulin as Cryo- and Lyoprotector of Bovine Plasma Proteins

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Additional information is available at the end of the chapter

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1. Introduction

Inulin is a generic term applied to heterogeneous blends of fructo-oligosaccharides [1] which are reserve carbohydrate sources present in many plant foods such as bananas, onions, garlic, leeks, artichokes and chicory, which represents the main commercial source. This polysaccharide has a wide range of both, nutritional and technological applications. Nutritionally, inulin is regarded as a soluble fiber which promotes the growth of intestinal bacteria, acting as a prebiotic. Also, is a non-digestible carbohydrate with minimal impact on blood sugar and unlike fructose, it is not insulemic and does not raise triglycerides being generally considered suitable for diabetics and potentially helpful in managing blood sugar-related illnesses [2-4]. Among the technological benefits, inulin is used as fat and sugar replacement, low caloric bulking agent, texturing and water-binding agent [5,6]. One general property of the saccharides is the stabilization of proteins by their incorporation into carbohydrate solutions before freeze-drying being this a known preservation procedure [7-10]. The previous incorporation of saccharide promotes the formation of amorphous, glassy systems, inhibits crystallization and influences the kinetics of deteriorative reactions upon storage by which its structured integrity is maintained [8,9,11,12]. To act successfully as a protectant, the saccharides should have a high glass transition temperature (T_g), a poor hygroscopicity, a low crystallization rate, containing no reducing groups. When freeze-drying is envisaged as a method of drying, a relatively high T'_g of the freeze concentrated fraction is preferable. Previous studies demonstrated that inulin meets these requirements being excellent protector of therapeutical proteins and viruses over the drying and storage processes [13,14].

The protein preserved by freeze-drying simplifies aseptic handling and enhances stability of protein products, with limited shelf lives in solution, by obtaining a dry powder without excessive heating. However, during the freeze-drying process the protein may lose its

activity and must be protected from conformational changes or denaturation [11,15]. The stabilization of proteins conferred by saccharides during freeze-drying has been explained by several mechanisms. First, replacing the hydrogen bonding between water and protein stabilizes the protein during drying processes, and second, the formation of a glass matrix where the protein is encapsulated avoiding its unfolding and thus preserving its conformation during freeze-drying [8,12,16-18]. Therefore, through the correct selection of the saccharide it is possible to improve the stability of proteins through their encapsulation in a glassy matrix, where molecular mobility is quite limited so that the rates of diffusion-controlled reactions, like protein unfolding or protein aggregation, are reduced [16,19,20].

Information about the energy of a protein can be obtained by means of thermal denaturation studies, allowing the characterization of their behavior during freeze-drying cycle. Differential scanning calorimetry (DSC) is one of the most useful methods for assessing protein thermal behavior and to obtain thermodynamic parameters of folding-unfolding transitions [21].

During the freeze-drying of a protein solution with or without saccharides to protect the structure, the 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 concentrate 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 the product collapse [20,22,23]. 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-rubber transition at a specific temperature which is named as the glass transition temperature (T_g) [24-28]. It is noteworthy that amorphous materials are stable in the glassy state below T_g , when the temperature is higher the viscosity decreases and thus the rate of chemical reactions increases and crystallization events occur, increasing the rate of deterioration during storage [22,25,27-29]. Both transitions T'_g and T_g are important parameters in the development of the freeze-drying cycle because not only ensures the stability and quality of the product, but also allow to improve the efficiency of the manufacturing process [20,22,28,30].

A diagram of phases for the water-saccharide system is shown in Figure 1. The curve of the freezing temperature separates the zones corresponding to the liquid and the solid (ice) solution phases. In fact, this procedure is aimed at obtaining a glassy system at room temperature as indicated in D. To get to this state, the freeze-dried process indicated by the curve A-B-C-D-E is carried out. The curve for the glass transition temperature (T_g) is reached when the solution is overcooled (B-C) until the T'_g in point C, where the concentration of the vitrification agent (saccharide) is given by C'_g . Then the water is eliminated and the solute concentration increases (C-D-E), obtaining a solid with an amorphous structure that exhibits a glass transition temperature (T_g) [22,28].

Therefore, the determination of the freeze-drying cycle is important because of physical changes that occur in the solution during the process, its study can be applied to improve processability, quality, and stability of the product during storage [29].

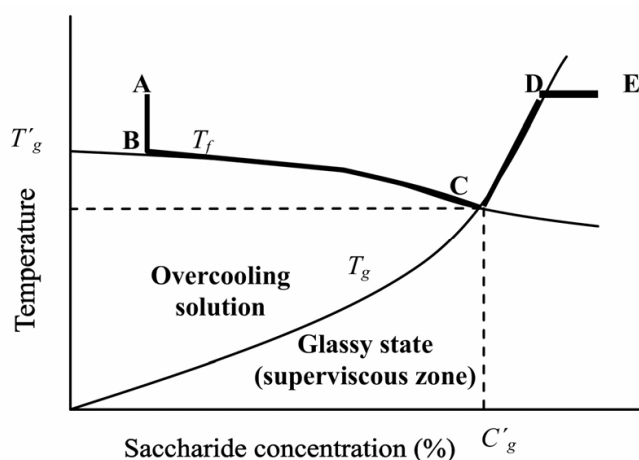


Figure 1. Phases diagram of the water-saccharide system. The curve A-B-C-D-E indicated the freeze-dried process. (T_f = freezing temperature; T_g = glass transition temperature; T'_g = glass transition temperature of the overcooling solution; C'_g = saccharide concentration)

Although many authors reported the use of saccharides as cryoprotectants of proteins and inulin as a good protector agent of some compounds, the present study is an attempt to evaluate inulin as cryoprotector of food proteins such as bovine plasma proteins, taking profit of the nutritional and technological benefit of the polysaccharide. Also there is a limited amount of data on glass transition temperatures for multicomponent mixtures and on the comparison of experimental and predicted values for such mixtures [28]. Then, the purposes of this study were *i)* to investigate the transition temperatures and the thermal denaturation of bovine plasma proteins stabilized with inulin in a glassy matrix in comparison to the effect of a monosaccharide (glucose) and a disaccharide (sucrose) at different concentrations using DSC, *ii)* to compare the quality, performance and storage conditions of these products.

The glass transition temperatures of the maximally concentrated frozen solutions (T'_g) were analyzed and compared to the experimental results by applying the predictive equations of Miller/Fox and Gordon/Taylor extended for multi-component systems. The glass transition (T_g) of the freeze dried multi-component mixtures, the onset crystallization temperature (T_c) of the solute at temperatures above T_g , in the freeze dried samples were determined. Furthermore, the kinetics of the denaturation and the thermal denaturation (T_d) of the freeze-dried samples, at different DSC scan rates, protein concentrations and pH, were analyzed and the thermodynamic compatibility of the different matrix components were determined. The enthalpy of change involved in the denaturation reactions of proteins (ΔH) was also determined. A kinetic model that describes bovine plasma proteins denaturation was proposed.

2. Materials and methods

2.1. Raw materials

The inulin used as cryoprotectant is mainly constituted by linear chains of fructose, with a glucose terminal unit, and has a molecular weight of 2400 g/mol. The commercial product

was provided by Orafit Chile S.A. and was obtained from chicory. The other saccharides employed to compare their performance were: *i*) a monosaccharide, glucose (Parafarm, Argentina), with a purity of 99.99% and *ii*) a disaccharide, commercial sucrose (Ledesma S.A., Argentina).

The protein used in the study was spray dried bovine plasma (Yerubá S.A. Argentina). The molecular weights of the proteins were in the range of 15.000 to 80.000 Da. The composition was $76 \pm 5\%$ proteins, $<0.1\%$ fat, 10% ash, 4% water, 1% low molecular weight compounds.

2.2. Preparation of Protein/carbohydrate samples: Concentration of bovine plasma proteins through ultrafiltration and freeze-drying treatments

The protein concentrate was obtained by means of a membrane process, which allowed protein concentration, eliminating insoluble macroscopic components, reducing the saline content [18]. The steps of the process were: *i*) the bovine plasma was dissolved in de-ionized water to a concentration of 3% w/v using a mixer at a low speed to avoid the formation of vortex and to minimize the appearance of foam; *ii*) the solution was passed through a porous support (Viledon FO 2431D, Germany) to remove macroscopic aggregates and reduce the saline content; *iii*) the feed solution (3 L) was thermostated in a water bath and impelled with a centrifugal pump, first through a frontal flow stainless steel filter, with a pore size of 60 μm (Gora, Argentina) (this procedure of microfiltration (MF) reduces the amount of bacteria and spores and acts as cold pasteurization, moreover this stage protects the ultrafiltration (UF) membrane from fouling); and finally, *iv*) the UF was performed using Pellicon cassette module (Millipore, Bedford, MA, USA), containing modified polyethersulfone membranes with a molecular weight cut-off (MWCO) of 10 kDa, with a membrane area of 0.5 m^2 . The concentration of proteins by UF was carried out by continuously removing the permeate stream until the desired concentration of 4% (w/v), was achieved. The experimental runs were performed at a transmembrane pressure (ΔP) of 1.5 bar, flow rate of (2.9 ± 0.05) L/min and a temperature of 10 $^{\circ}\text{C}$. Additionally a discontinuous diafiltration (DD) process was applied to removal salts and other contaminant of low molecular weight. For this operation the starting solution was the UF concentrate, which was diluted to the initial volume (3 L) with de-ionized water in a single state and ultrafiltered to the desired concentration range.

The UF membrane undergoes a fouling process during protein permeation so a cleaning protocol may be applied. It was performed by applying a "Cleaning in Place" (CIP) procedure according to the manufacturer's instructions. At the end of each run, a cycle of water/ alkali (NaOH , $\text{pH} = 12.5 \pm 0.5$)/ water wash was applied to the membrane at (40 ± 2) $^{\circ}\text{C}$ and at a transmembrane pressure of 1 bar. Furthermore, a cleaning step using NaClO (commercial grade) 300 ppm was carried out at the same temperature and pressure to ensure sanitation and cleaning. Measurements of normalized water permeability were performed in order to verify recovery of flow through the membrane which ensures the recuperation of membrane permeability.

The bovine plasma protein (BPP) concentrate obtained by UF (concentration: 4 % w/v) was fractioned: A fraction as witness sample was reserved and the protective agent (glucose,

sucrose, 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 $-40\text{ }^{\circ}\text{C}$ and freeze-dried using a lyophilizer (Rificor S.A., Argentina) at 1 bar for 48 h. The samples temperature was controlled by a temperature sensor. The denatured protein content was determined before and after the freeze-drying.

2.3. Differential Scanning Calorimetry (DSC) measurements

Determination of T'_g in the protein solutions

The solutions containing plasma proteins–saccharides mixture were analyzed to determine T'_g at different pH values and saccharide concentrations by DSC with a Q100DTA Instrument (USA). The pH was adjusted using 0.1 N of NaOH and HCl. Protein concentrate solutions (average composition: saccharide 5% p/v - protein 4% p/v; saccharide 10% p/v - protein 4% p/v; saccharide 15% p/v - protein 4% p/v), ($10 \pm 2\text{ mg}$) 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. Samples Solutions were: (a) equilibrated at $20\text{ }^{\circ}\text{C}$ and held for 1 min; (b) cooled at $2\text{ }^{\circ}\text{C}/\text{min}$ until $-80\text{ }^{\circ}\text{C}$ for glucose, $-60\text{ }^{\circ}\text{C}$ for sucrose and $-40\text{ }^{\circ}\text{C}$ for inulin and held for 30 min; (c) warmed up to the annealing temperature (-50 , -40 and $-20\text{ }^{\circ}\text{C}$, for glucose, sucrose and inulin, respectively) by employing an annealing time of 30 min at heating rate of $2\text{ }^{\circ}\text{C}/\text{min}$ [31]; (e) recooled at the same temperature of step (b) and held for 30 min; (f) warmed up to $0\text{ }^{\circ}\text{C}$ at heating rate of $2\text{ }^{\circ}\text{C}/\text{min}$. 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 melting.

Determination of T_g , T_c and T_d of proteins in the freeze–dried solids

Heat induced conformational changes on freeze-dried bovine plasma protein concentrate (BPP concentrate) in the amorphous carbohydrate matrix. The freeze-dried solids were analyzed to determine T_g , T_c and T_d at different pH values and saccharide concentrations by DSC with a Q100DTA Instrument (USA). The pH was adjusted using 0.1 N of NaOH and HCl. Protein concentrates (average composition: freeze-dried with saccharide 5% (p/v) = saccharide 35% p/p - protein 55% p/p; freeze-dried with saccharide 10% (p/v) = saccharide 64% p/p - protein 28% p/p; freeze-dried with saccharide 15% (p/v) = saccharide 79 % p/p - protein 14% p/p), ($12.5 \pm 2.5\text{ mg}$) 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.

Freeze–dried solids were equilibrated at $0\text{ }^{\circ}\text{C}$, held for 1 min and then warmed up to $200\text{ }^{\circ}\text{C}$ at heating rate of $2\text{ }^{\circ}\text{C}/\text{min}$. To check the irreversibility of the reaction of heat-induced conformational changes, the samples after the end of the first heating stage described before, were re-scanned. For this, the protein-saccharide samples were cooled to $20\text{ }^{\circ}\text{C}$ and stabilized during 5 min, and then warmed up to 200°C . Samples of freeze dried bovine plasma protein concentrate (BPP concentrates) in the amorphous carbohydrate matrix at pH

8, 6 and 4, at different heating rates of 2 and 5 °C/min in the temperature range 20–200 °C were analyzed. The pH was adjusted using 0.1 N of NaOH and HCl. Measurements were carried out on three separate samples (replicates). The following parameters were calculated at least in triplicate: T_d , at maximum heat flow, and ΔH , the enthalpy change involved in the overall heat-induced reactions within the protein molecules, that was determined by integrating the area beneath the enthalpy peak and above a straight baseline drawn in between the beginning and the end of the transition temperature range [32-34]; 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 [22,32,33].

2.4. Determination of native protein content

The native protein content is a measure of protein functionality preservation. It was determined after isoelectric precipitation of denatured/aggregated protein [18,35]. Dispersions of protein concentrate at 1% (w/v) were adjusted to pH value inferior of the pI of plasma proteins (~ 4.8) using 0.1 N of NaOH and HCl. An aliquot of the solution was centrifuged in a refrigerated ultracentrifuge (Beckman J2-HS) at 20,000 rpm 30 min at 5 °C. Protein concentration in the supernatants was diluted in a dissociating buffer (EDTA 50 mM, urea 8 M, pH= 10) and determined by molecular absorptiometry at 280 nm. The results were reported as percentage of the total protein concentration [36]. The percentage of native protein content of suspensions at pH 4.8 was obtained as the ratio between soluble protein (SP) and total protein (TP) contents after aggregation of denatured protein (Eq. 1).

$$NP\% = \left(\frac{SP}{TP} \right) \times 100 \quad (1)$$

2.5. Scanning electron microscopy

The microstructure of freeze-dried plasma concentrates with and without saccharides was analyzed by scanning electron microscopy (SEM) using an LEO1450VP equipment (Zeiss, Germany). Powder samples were mounted on double-sided carbon adhesive tape on aluminum stubs and gold-coated and processed in a standard sputter. The micrographs were obtained in high vacuum at 10 KeV.

2.6. Statistical analysis

The experimental data were statistically analyzed by the Tukey-Kramer multiple comparison test, in the cases where 2 or more comparisons were considered, assuming that

a $P < 0.05$ was statistically significant [37]. Statistical GraphPad InStat software (1998) was used.

3. Theoretical considerations

3.1. Equations for T_g prediction

The Miller/Fox equation can be used for the determination of T_g dependence with the composition in a multi-component system, assuming constant density of the solutions, independent of temperature [28,38,39]. For a ternary mixture (protein-saccharide-water), it can be written as:

$$\frac{1}{T_g} = \frac{m_1}{m_t T_{g1} (\rho_1 / \rho_t)} + \frac{m_2}{m_t T_{g2} (\rho_2 / \rho_t)} + \frac{m_3}{m_t T_{g3} (\rho_3 / \rho_t)} \quad (2)$$

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 [40] 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 [41,42]. Instead we proposed a system considering each individual component: bovine protein concentrate, saccharide and water, with each corresponding property [43]:

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2} + k^2 w_3 T_{g3}}{w_1 + k w_2 + k^2 w_3} \quad (3)$$

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 the software Excel 2003 (Microsoft).

Eqs. (2) and (3) were used for the determination of T_g of the frozen solutions.

3.2. Theory of protein unfolding

Unfolding of protein is suggested to involve at least two steps according to Lumry and Eyring model (1954). The first step is a reversible unfolding of the native protein (N). This is followed by an irreversible change of the denatured protein (D) into a final irreversible state (I) [44,45].



A special case was when $k_2 \gg k_{-1}$, where most of the D molecules will be converted to I as an alternative to refolding back to the native state. In this case, the denaturation process can be regarded as one-step process following first-order kinetics [44-46], (Eq.5).

$$N \xrightarrow{k} I \quad (5)$$

where the first-order rate constant k can be identified with k_1 of Eq. (4). The total absorbed heat now equals the enthalpy change from N to I ; it was generally assumed that the enthalpy change from D to I was negligible compared to that from N to D [44].

Experimentally, the irreversibility of unfolding was verified in a rescans. For an irreversible process, in the DSC rescanned thermograms no transition could be observed.

4. Results and discussion

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

As was previously mentioned, to avoid collapse of the products during the freeze-dried process, a temperature below the glass transition temperature of the frozen concentrated solutions, must be attained. Inulin as protein protective agent was comparatively studied, employing mono and disaccharides. The thermograms of Figure 2 show the transition temperatures of the frozen solutions of bovine plasma with inulin compared to the other saccharides, obtained in a single scan.

The result indicated that at each saccharide concentration, T'_g was higher for inulin (Table 1), suggesting that it has a greater cryostabilizing effect on bovine plasma proteins than the other saccharides, improving product stability. It was also observed that T'_g increased with the molecular weight of the cryoprotectant that is: inulin > sucrose > glucose. The same tendency was reported previously by means of the evaluation of protein shelf life time [18]. By the other hand, it was reported that inulin exhibit better stabilizing properties than sucrose and trehalose in the prevention of the nonPEGlated lipoplexes aggregation [14]. Many studies concluded that transition temperatures increased with the saccharides molecular weight [22,23,27]. For example T'_g of freeze-dried surimi depended strongly on the type and content of sugar and at each sugar level the T'_g was trehalose > sucrose > glucose > sorbitol [47].

Thermograms of bovine plasma solutions revealed the existence of two glass transitions (T'_{g1} and T'_{g2}) for glucose and sucrose as protective agents, evidenced as deviations in the base line (indicated by arrows in Figure 1). Similar results were found by Telis and Sobral [48] who worked with freeze-dried tomato. This may be because the presence of phases formed by different proportions of saccharide, water, and proteins present in the frozen solution [47-49]. Also it was observed that when the saccharide concentration increased, T'_{g1} and T'_{g2} increased and decreased respectively (Table 1). However a constant average value was maintained between both T'_g values for each sugar, being -51.2 ± 0.8 and -41.1 ± 0.1 for glucose and sucrose, respectively. Similar results were found in [47] on freeze-dried surimi product with trehalose. For inulin only one T'_g was found, which increased with the increase of saccharide concentration. From these results and considering that the water acts as plasticizer, i.e. decreases drastically T'_g of food polymers [26], it can be concluded that the conditions of the freeze-drying process, are linked directly to T'_g of the frozen solution.

Therefore, it is important to note that the higher value of T'_g observed in frozen solutions with inulin, allowed higher freezing temperatures during processing reducing production costs.

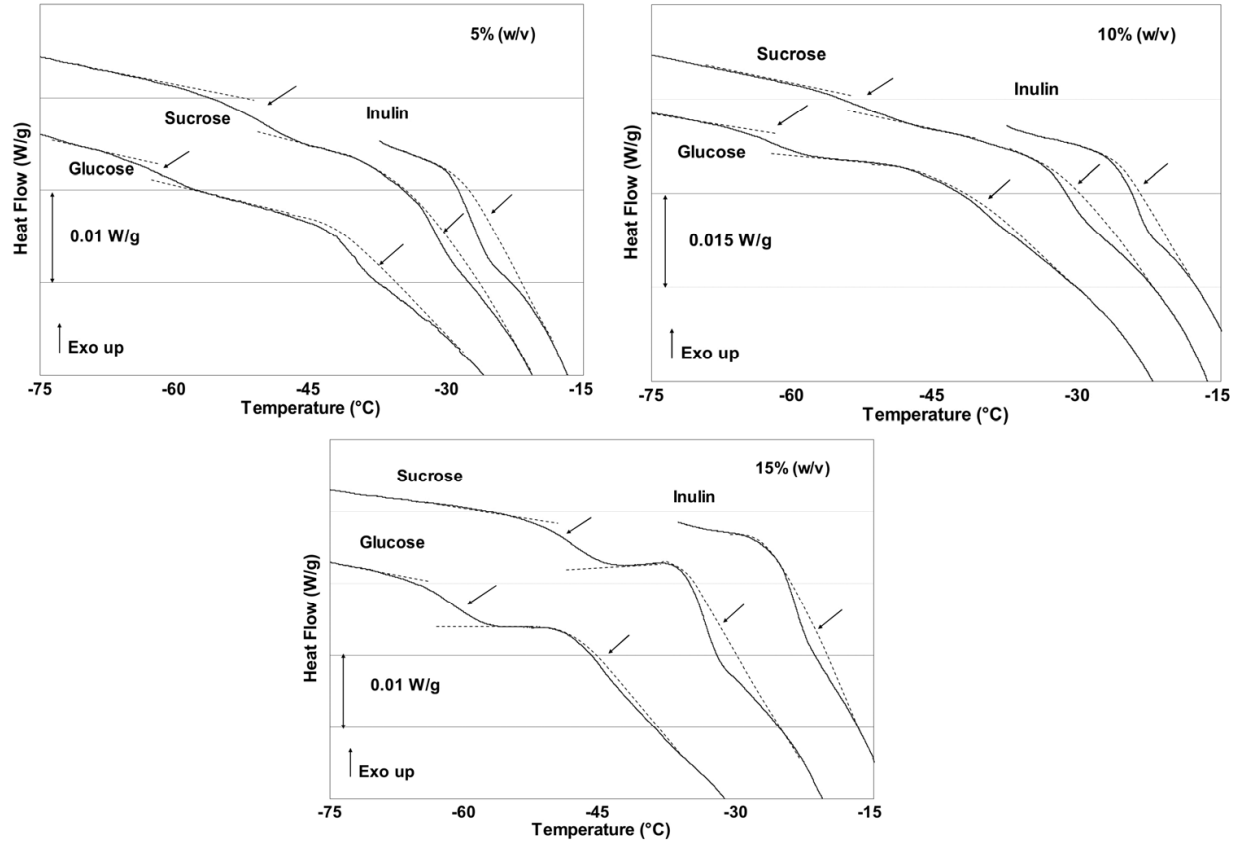


Figure 2. DSC thermograms for freeze bovine plasma protein-saccharide solutions. Down-arrows indicate T'_g . Scan rate = 2°C/min ; pH = 8.

Saccharide	Concentration (% w/v)	T'_{g1} (°C)	T'_{g2} (°C)	T_g (°C)
Glucose	5	-62.50 ± 0.58^a	-39.24 ± 0.75^a	16.31 ± 0.38^a
	10	$-61.06 \pm 0.45^{a,b}$	-39.91 ± 0.83^a	41.52 ± 0.29^b
	15	-59.82 ± 0.68^b	-44.96 ± 0.49^b	60.31 ± 0.48^c
Sucrose	5	-51.48 ± 1.05^c	-31.15 ± 0.40^c	48.01 ± 0.56^d
	10	$-50.12 \pm 1.03^{c,d}$	-31.86 ± 0.60^c	52.48 ± 0.52^e
	15	-48.42 ± 0.98^d	-33.72 ± 0.45^d	64.28 ± 0.46^f
Inulin	5	-26.96 ± 0.68^e	-	48.85 ± 0.35^d
	10	-23.67 ± 0.55^f	-	66.18 ± 0.69^g
	15	-22.40 ± 0.45^f	-	69.25 ± 0.45^h

Table 1. Effect of type and concentration of cryoprotectant on glass transition temperature (T'_g) and lyoprotectant on glass transition temperature (T_g) of freeze bovine plasma proteins solutions (heating rate: 2 °C/min). Values represents the means \pm standard deviation; n = 3. Values followed by different letters in the same column are significantly different from each other ($P < 0.05$).

The effect of water as a plasticizer of the mixture protein-saccharide was predicted by the Miller/Fox and Gordon–Taylor equations, the results, were compared with experimental values (Table 1). The data of T'_g of all pure components required for the Eq. (1) are listed in Table 2.

The densities of bovine plasma proteins, glucose, sucrose and inulin (at room temperature) were determined with a digital densimeter, and the results were: $0.4 \pm 0.08 \text{ g/cm}^3$, $0.6 \pm 0.05 \text{ g/cm}^3$, $0.8 \pm 0.04 \text{ g/cm}^3$ and $0.3 \pm 0.05 \text{ g/cm}^3$, respectively.

From literature the T_g of the water is -135°C [41] and the T'_g of plasma protein is $-11 \pm 2^\circ\text{C}$ [22]. The T_g value of bovine plasma protein for Eq. (3), was $65 \pm 3^\circ\text{C}$. Entering this data into Eqs. (2) and (4), the predicted values of T'_g were obtained, which are listed in Table 3. 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 is defined as the resistance to a T'_g decrease induced by the plasticizing effect of water [26,41,47]. The order found for k value of the saccharides was: inulin > sucrose > glucose. Although the highest value of k is for inulin, this saccharide has the highest T_g value, allowing a greater value T'_g and therefore generating a lower cost during processing, preventing also the collapse of the product at temperatures relatively higher during the freeze-drying.

Saccharide	T'_g ($^\circ\text{C}$)		
	5 % (w/v)	10% (w/v)	15 % (w/v)
Glucose	-85	-79	-72
Sucrose	-59	-53	-46
Inulin	-17	-15	-13

Table 2. Data from references used in the calculation of T'_g by Miller/Fox and Gordon/Taylor modified equation [18].

	Glucose %(w/v)			Sucrose %(w/v)			Inulin %(w/v)		
	5	10	15	5	10	15	5	10	15
$^*\rho$ (g cm^{-3})	1.039	1.042	1.059	1.033	1.041	1.056	1.032	1.039	1.049
T'_g ($^\circ\text{C}$) (Miller/Fox)	-63.99	-60.46	-56.1	-54.07	-51.24	-47.04	-30.43	-23.64	-19.51
Difference (%)	2.32	0.99	6.63	4.79	2.18	2.95	11.40	0.12	14.8
T'_g ($^\circ\text{C}$) (Gordon/ Taylor modified)	-62.69	-61.26	-60.03	-51.38	-50.58	-48.47	-26.70	-24.23	-22.11
Difference (%)	0.30	0.33	0.35	0.19	0.91	0.10	0.97	2.31	1.31
k	3.5			4.1			4.5		

Table 3. Glass transition parameters for the multicomponent system: plasma bovine proteins-saccharides-water. $^*\rho$: solution density ($T=19.8^\circ\text{C}$)

4.2. Effect of saccharides on glass transition of the freeze-dried samples

The storage temperature of frozen or freeze-dried foods should be below the glass transition temperature as previously established [22,27,42,50]. Figure 3 shows the thermograms of the freeze-dried samples containing inulin compared with glucose and sucrose at different concentrations. The existence of these transitions evidenced the glassy state of the freeze-dried plasma protein/saccharides mixtures. Besides, Table 1 shows that T_g of the sample increases with increasing saccharide concentration. Similar results were found in the references [28,30]. This effect can be explained considering that sugar forms hydrogen-bridge bonds with proteins reducing the available volume for the interaction with water molecules, so water become less effective as plasticizer with an increase in saccharide content [51]. Also was observed that T_g of the freeze-dried samples increased with increasing of the molecular weight of the cryoprotectant. Processes of devitrification and hence product spoilage can occur if the temperature of storage is higher than the T_g of the sample. Therefore, the higher T_g value of inulin provides greater stability at higher temperatures, reducing the storage costs.

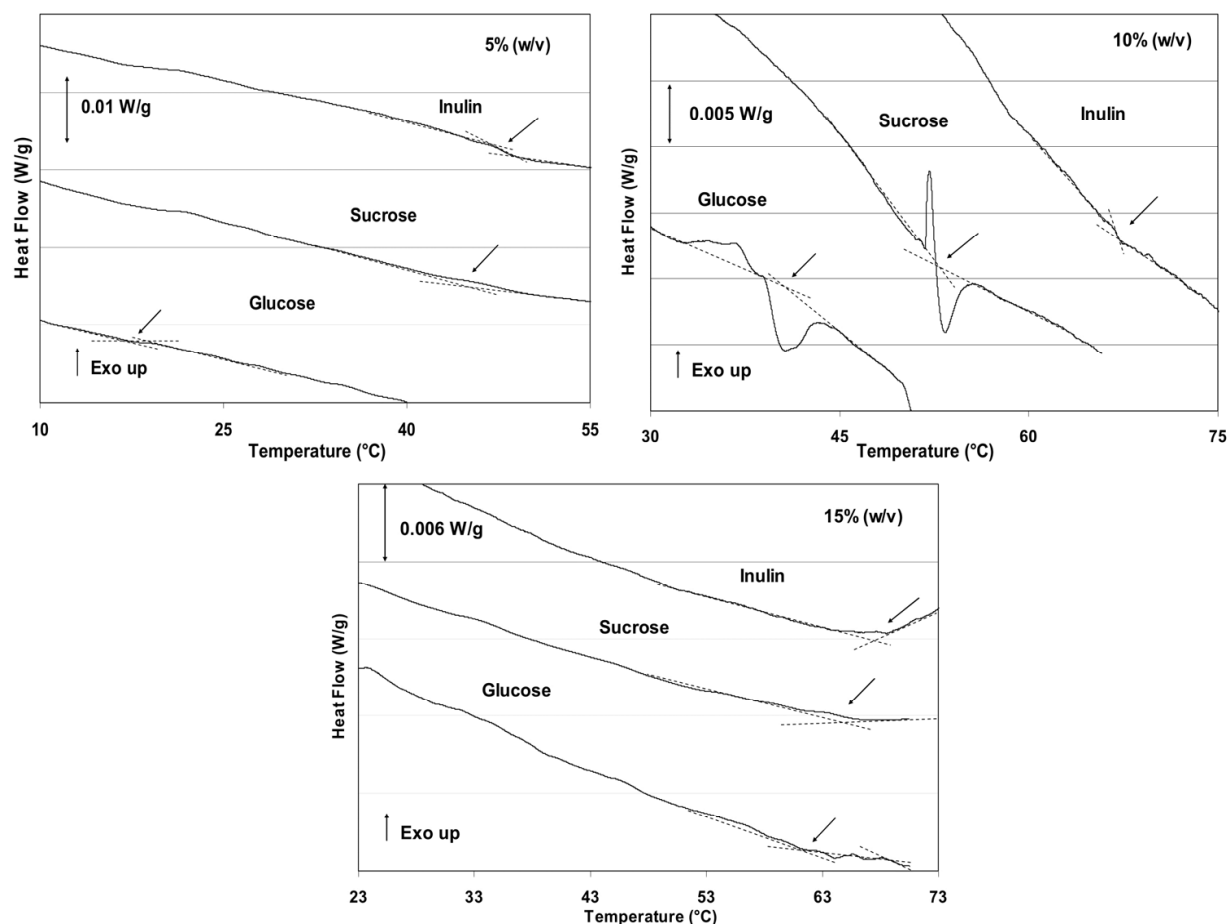


Figure 3. DSC thermograms for freeze-dried bovine plasma protein-saccharide mixtures. Down-arrows indicate T_g . Heating rate: 2°C min⁻¹; pH=8

4.3. Effect of saccharides on crystallization temperature of the freeze-dried samples

It is important to determine the crystallization temperature (T_c) of the freeze-dried samples since crystallization causes the most drastic changes on physical properties of food polymers and affects considerably food stability. The glass transition is often followed by crystallization of the solutes where the molecular mobility increases and the sample crystallizes increasing the rate of food spoilage [27,28,30].

Fig 4 shows 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) estimated as the area under the peak for the different protective agents at different concentrations. The crystallization temperature of freeze-dried samples was found to depend on the molecular weight and the saccharide concentration [27,30]. Therefore, the results showed that the presence of inulin at the same concentration than the other saccharides further increases the T_c value of freeze-dried solutions. Mixtures containing a saccharide concentration of 10 % (w/v) show an increase of ΔH_c , indicating a higher amorphous content. This behavior can be explained considering that a suitable proportion of saccharide and protein in the mixture allows a better interaction among these components [51,52,43].

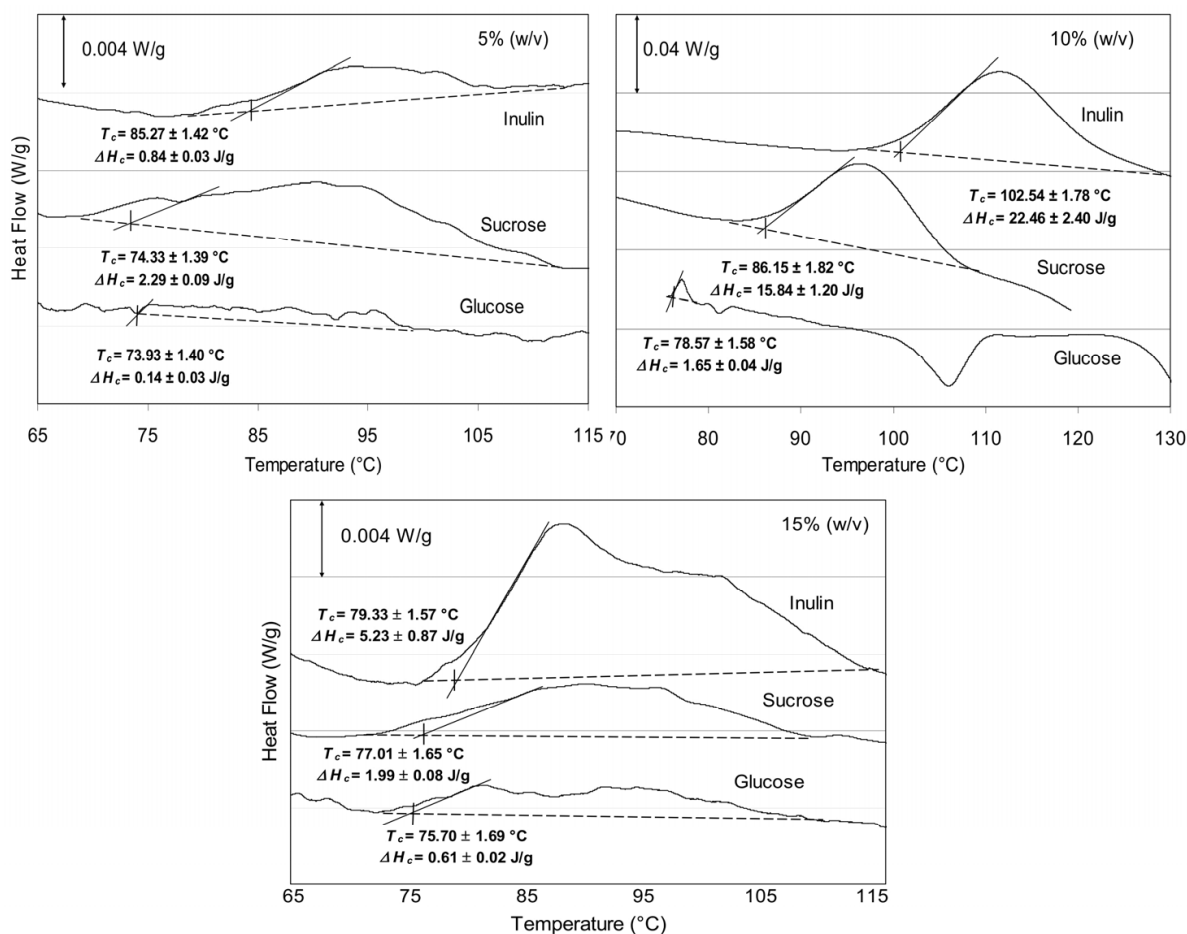


Figure 4. DSC thermogram for freeze-dried bovine plasma protein with the protective agents at different concentrations. The exothermic event indicates T_c . Heating rate: 2°C min^{-1} ; pH=8.

4.4. Thermal denaturation of BPP in a matrix of saccharide

4.4.1. Effect of saccharide type and concentration

The thermal stability of BPP in a matrix of inulin compared with other saccharides was investigated using DSC. Table 4 shows the values of T_d obtained for BPP concentrate without protective agents and in different matrixes of glucose, sucrose and inulin at different concentrations. The value of T_d for BPP concentrate (88.19 ± 1.87 °C), was obtained from thermograms without protective agent and was similar to that reported in reference [53], for blood plasma. Comparing this value with the protein sample immersed in a matrix of saccharide, it was observed an increase in the value of T_d in all the cases, indicating a higher thermal resistance due to the stabilizing effect of saccharides. A similar behavior was observed in the DSC study of whey protein concentrates with the addition of honey [54]. Evaluating among the saccharides at the same concentration, it can be concluded that the higher the molecular weight of the carbohydrate, the higher was the T_d , thus inulin > sucrose > glucose. This behavior was in agreement with that reported in [55], in multi-block copolymers. With respect to the range of saccharide concentrations studied, optimum concentration was 10% (w/v), as it is shown in Table 4, in terms of the values of T_d and ΔH .

Saccharide	Concentration (% w/v)	T_d (°C)	ΔH (J g ⁻¹)
Glucose	5	110.07 ± 1.22^a	0.84 ± 0.32^a
Sucrose		132.78 ± 2.12^b	$5.08 \pm 0.98^{b,c}$
Inulin		143.81 ± 0.89^c	$2.97 \pm 0.55^{a,d,c}$
Glucose	10	$107.27 \pm 0.85^{a,d}$	12.26 ± 0.92^e
Sucrose		144.95 ± 2.34^c	22.40 ± 1.23
Inulin		156.21 ± 1.12^e	12.22 ± 1.43^e
Glucose	15	104.91 ± 0.89^d	$3.77 \pm 0.98^{d,f}$
Sucrose		126.66 ± 1.54^f	7.01 ± 1.22^b
Inulin		132.57 ± 1.34^b	$5.78 \pm 0.76^{b,f,c}$

Table 4. Effect of saccharide concentration on the denaturation temperature of freeze dried BPP concentrate. Heating rate: 2°C min⁻¹. pH=8. Values followed by different letters in the same column are significantly different from each other ($P < 0.05$).

The functional structure of a protein in solution is determined by electrostatic forces, hydrogen bonds, Van der Waals interactions and hydrophobic interactions. All these interactions are influenced by water, becoming essential for the functional unfolding of most of the proteins. As water is eliminated during freeze-drying, peptide-peptide interactions prevail causing an alteration in the secondary, tertiary or quaternary structure of the protein, i.e. a conformational change of it. However, the presence of sugar displaces and supplants water forming hydrogen bonds with the dry protein which maintains its structured integrity into the glass matrix. In the case that the formation of the glass structure did not occur, the sugar would be excluded and it would not be available for the formation of hydrogen bonds to protect the dry protein from its unfolding or loss of conformation [13,14].

The protective effect of saccharides depends on its concentration, since as the concentration increases there are more possibilities of forming hydrogen bonds with the protein [11,18]. However, when concentrations were higher than 10 % (w/v), a lower protection was obtained. This result can be explained taking into account that at high concentrations, the saccharide starts to crystallize during freeze-drying, being prevented the formation of hydrogen bonds with the dry protein [12]. This behavior was confirmed by determination of the native proteins in the protein-saccharide matrixes employing eq. (1). The results are presented in Figure 5, which shows that there is a maximum at a concentration of 10% (w/v) for the different saccharides analyzed, indicating higher protein protection and stability.

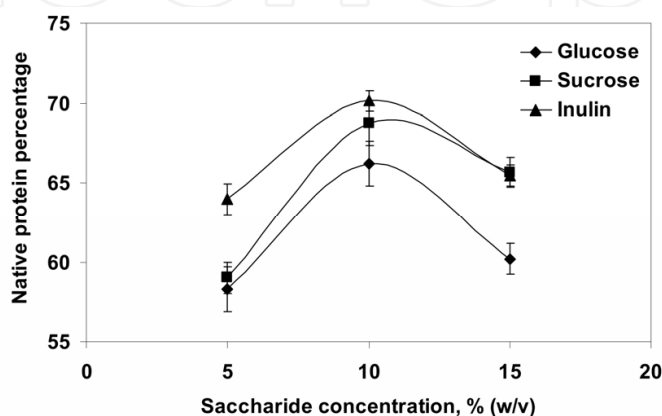


Figure 5. Native protein percentage of freeze dried BPP concentrate with different protective agents at different concentrations.

4.4.2. Effect of pH

To determine the application of these formulations is important to know the variation of T_d as a function of pH due to the wide range of environmental conditions existing in food. Table 5 shows the T_d values of BPP concentrate in a glassy matrix of saccharides at different pH values.

Saccharide	pH	T_d (°C)	ΔH (J g ⁻¹)
Glucose	8	107.27 ± 0.85 ^a	12.26 ± 0.82 ^a
Sucrose		144.95 ± 1.34 ^b	22.40 ± 0.97 ^b
Inulin		156.21 ± 1.12 ^c	12.22 ± 0.55 ^a
Glucose	6	102.94 ± 1.33 ^d	34.74 ± 0.92 ^c
Sucrose		134.56 ± 2.16 ^e	43.15 ± 1.23 ^d
Inulin		152.98 ± 1.52 ^{c,f}	42.95 ± 1.45 ^d
Glucose	4	101.74 ± 1.27 ^d	9.58 ± 0.98 ^{a,e}
Sucrose		107.67 ± 1.56 ^a	9.32 ± 0.72 ^e
Inulin		151.84 ± 1.89 ^f	9.35 ± 0.96 ^e

Table 5. Effect of pH and addition of saccharides on the denaturation temperature of BPP concentrate. Heating rate: 2°C/min. Values represents the means ± standard deviation; n = 3. Values followed by different letters in the same column are significantly different from each other ($P < 0.05$).

With increasing alkalinity of the medium there is an increase in the values of T_d for each saccharide (pH 8), indicating that BPP concentrate was more stable at higher pH. Similar results were found in previous works in porcine blood plasma proteins and whey protein concentrate [34,54]. Comparing between different saccharides at the same concentration, it can be seen that inulin presents a higher T_d in all the pH range. The maximum ΔH values were observed at pH 6 indicating a higher amount of native protein. Similar ΔH values at pH = 6 were reported by Dàvila in reference [34]. The lowest values of T_d and ΔH were found at pH 4, this may be to the proximity with the isoelectric point of proteins (pI: 4.8-5.8), thus decreasing the electrical net charge and facilitating aggregation reactions.

4.4.3. Effect of scanning rate

The protein-saccharide mixtures were studied at different scanning rates (2 °C/min and 5 °C/min). As an example Figure 6 shows the transition temperature and enthalpy for sucrose at 10 % (w/v).

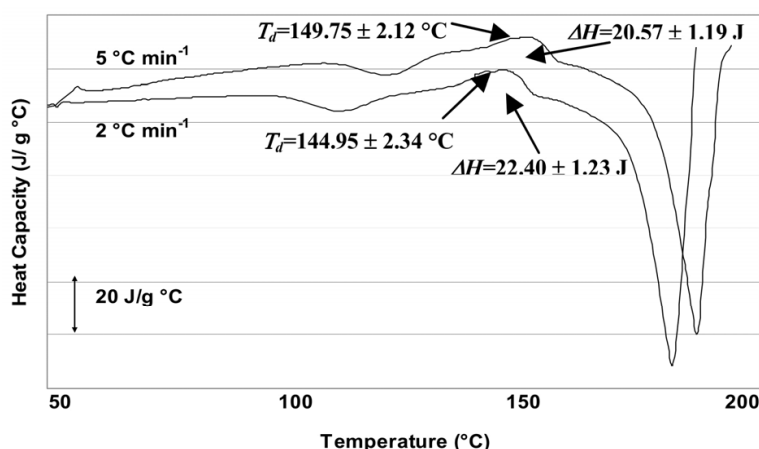


Figure 6. Effect of DSC heating rate on T_d values of freeze-dried BPP with sucrose 10%(w/v).

It was found for all the saccharides that T_d and ΔH are scanning rate dependent. T_d values increased 5 ± 2 °C in all the samples with increasing scanning rate, similar behavior was reported in references [56-58]. Furthermore, the ΔH decreased ($\sim 10\%$) with increasing scanning rate that was in agreement with the results reported in references [21,59]. Thus, the system was scanning rate dependent and so the thermal denaturation process was under kinetic control [33,44].

4.4.4. Study of Irreversibility of the Thermal Denaturation of BPP

The irreversibility of BPP denaturation was investigated by a multiple reheating experiment, according to the method proposed by Idakieva and Michnik [45,60]. From the initial DSC scan, we have determined the values of the transition temperatures at 107°C, 145 °C and 156 °C for glucose, sucrose and inulin at 10% w/v, respectively (Table 5). DSC tests were carried out as successive scans, where the heating was carried out up to different final temperatures, with a cooling up to 20°C between scans (Figure 7).

For glucose, sucrose and inulin, the first heating was carried out up to 75°C, and 85°C (temperatures below the T_d for all the saccharides), respectively; no thermal effect was observed in the thermal denaturation peak during the reheating experiment. However, if the rescanning was stopped over their transition temperatures, the endothermic peak of T_d disappeared completely. Therefore, the endothermic peak of T_d disappeared completely upon rescanning the sample at temperatures above T_d ; furthermore, as was previously described, the thermograms were scanning-rate dependent, suggesting both results that it was an irreversible event [61]. Similar behavior was also found for whey protein in an amorphous carbohydrate matrix [49], porcine blood plasma proteins [34] and BSA [33]. Irreversible denaturation of bovine plasma proteins might be due to processes such as aggregation, where hydrophobic interactions occur, and exposed thiol groups can form disulfide bonds, which result in an irreversible behavior [33]. Considering the Arrhenius law and the treatment developed in reference [43], the determination of the activation energy can be achieved from the experimental data. The obtained values were: 10443 J mol⁻¹, for BPP without protective agent; 27216 J mol⁻¹, 32058 J mol⁻¹ and 42099 J mol⁻¹ for BPP with glucose, sucrose and inulin, respectively, all of them at 10% (w/v). The results showed that

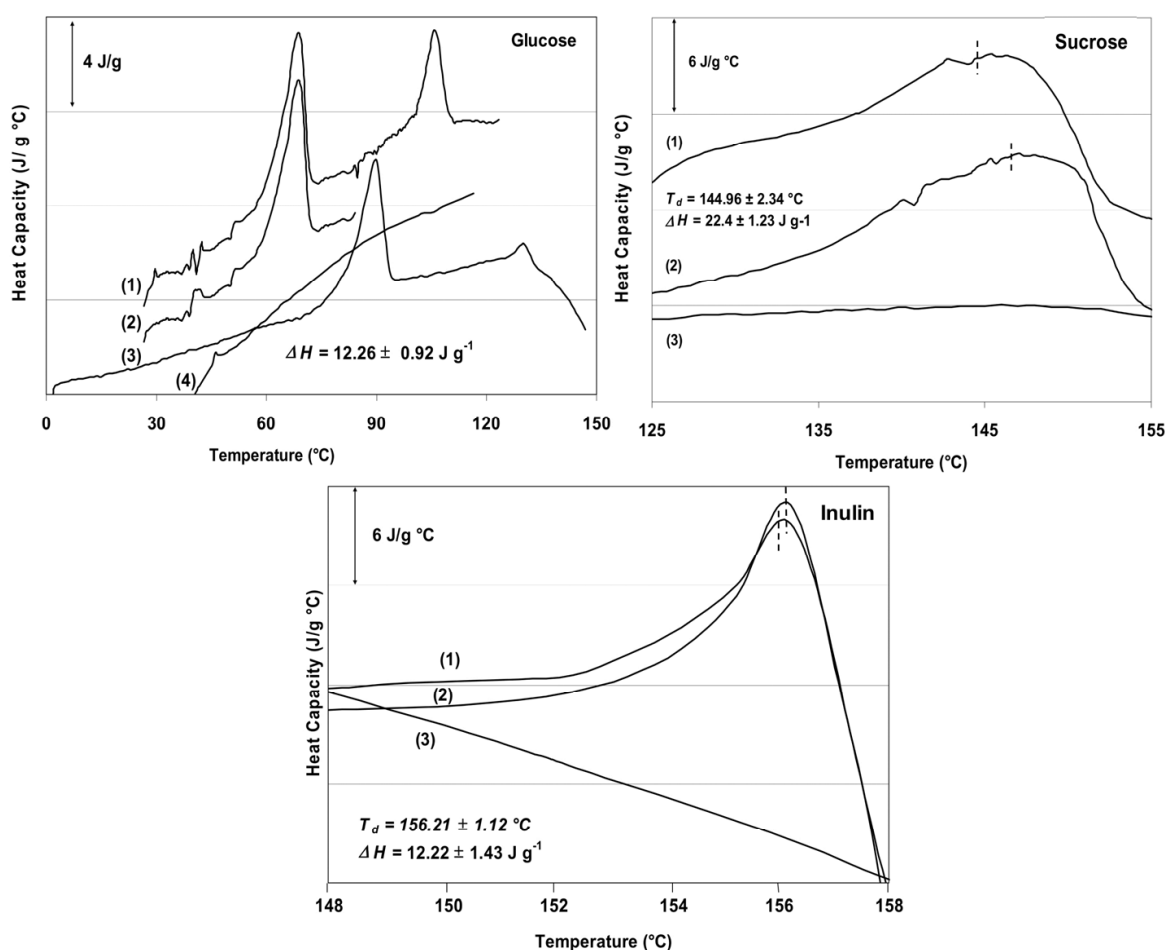


Figure 7. DSC thermograms of freeze dried BPP concentrate with saccharide at 10 % (w/v). DSC scans (2), (3), (4) represent thermograms from repeated heating and subsequent cooling. Scan (1) is a full scan to 120 °C (glucose), 155 °C (sucrose) and 158 °C (inulin).

with the addition of protective agents the activation energy increased; besides with increasing molecular weight, the activation energy also increased. Therefore, the addition of saccharides, especially of inulin caused a decrease in the rate of degradation reactions, obtaining a higher stabilization upon storage [8,14,18].

4.4.5. Study of the blends morphology through SEM

Figure 8 showed the SEM micrographs of blends of protein-saccharides.

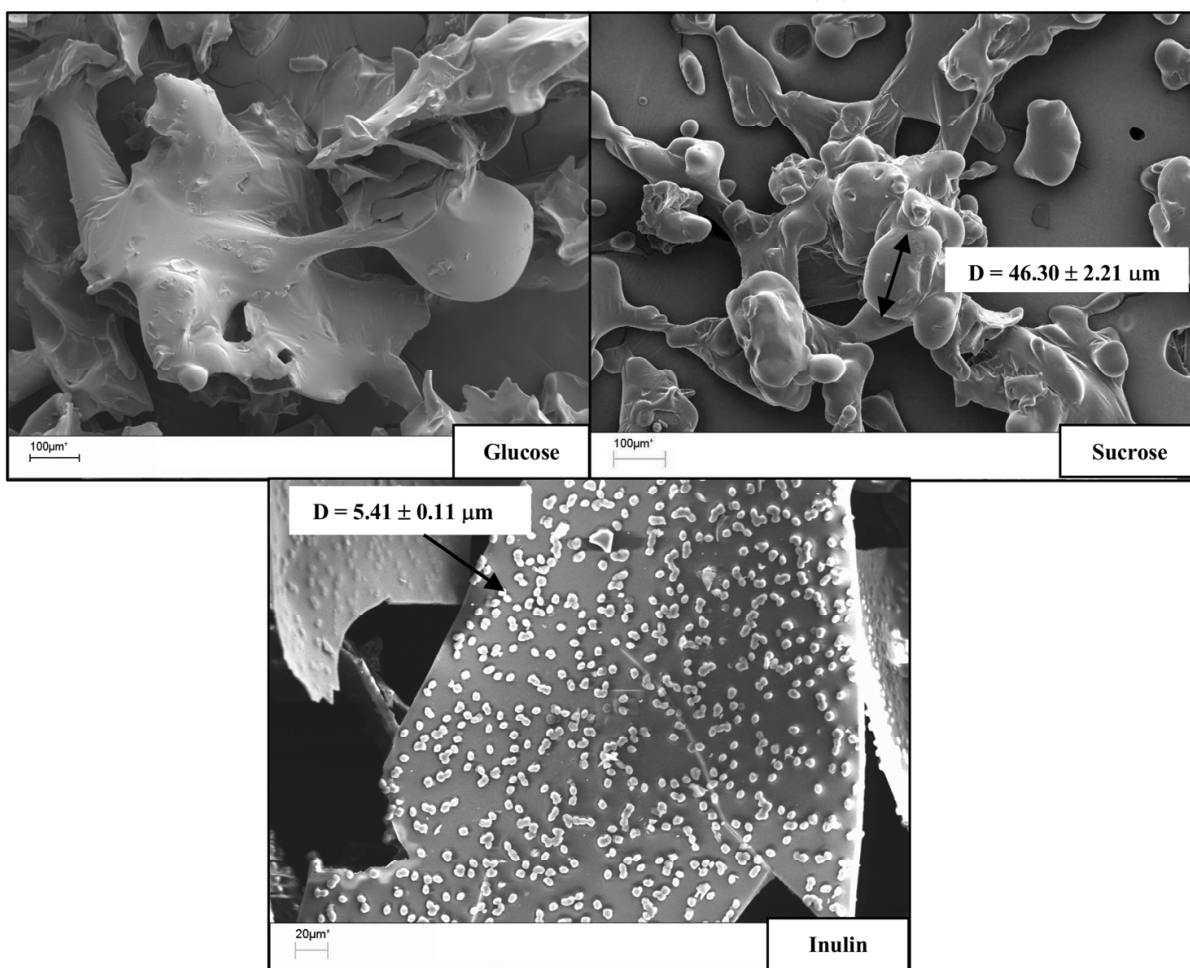


Figure 8. Scanning electron micrographs of the freeze-dried product with different saccharides, with a magnification of 200X for glucose and sucrose, 300X for inulin.

It was observed phases homogeneously distributed, indicating miscibility of the component in the matrix. The shapes were uniform, which was an attribute, linked with thermodynamic compatibility [62]. Based on the data previously obtained, comparing the transitions of the blends with respect to the value of the individual components, showed an

increase in the value T_d . This increase in the T_d values can be attributed to greater miscibility of the components of the mixtures, confirming what was observed in the micrographs [61]. Therefore, these results are in agreement with the concept of miscibility, which is based on the variation of the thermal behavior with respect to the individual materials [63].

5. Conclusions

The thermodynamic properties of the solution and the freeze-dried bovine plasma proteins–saccharides mixtures were investigated in this study. The DSC thermograms demonstrated that the bovine plasma proteins– inulin mixtures have the highest glass transition temperature for the protein solution and also the highest glass transition and denaturation temperature for the freeze-dried powder, optimizing the freeze-drying process and also stabilizing and protecting the proteins during storage in 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 saccharide 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. Also was found that 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. The addition of saccharides allowed the increase of the protein denaturation temperature and enthalpy, with an optimal saccharide concentration of 10% (w/v) and a pH range between 6 and 8. This change in the thermal properties shows a greater compatibility of the blends with 10% (w/v) saccharide, because this concentration causes the greatest changes in the values of T_d when compared with individual values of BPP. The results were corroborated by the SEM micrographs, showing homogeneously distributed phases, and denoting the highest miscibility between them. The temperature of thermal denaturation was scan rate dependent, and no thermal transition was detected in the re-scan experiments so it was concluded that the protein unfolding was irreversible and was adequately interpreted by the theoretical model employed.

Therefore, the results showed highest values of T_g and T_d in the freeze-dried samples of inulin proven that this compound is a better protein protective agent during storage than mono and disaccharides such as glucose and sucrose. In this way prevent the unfolding of bovine plasma proteins submitted to higher temperatures. Furthermore, the higher T'_g of frozen solutions of bovine proteins with inulin allows higher freezer temperatures during freeze-drying, reducing costs in a food elaboration. The finding about the inulin cryoprotant role of food proteins is relevant considering that it is a soluble fiber, categorized as a prebiotic, and being a valuable alternative as a functional ingredient for food formulation [64,65].

The findings regarding the protective effect of inulin on bovine plasma proteins, suggest that may be interesting the study of the behavior of formulated foods elaborated with the analyzed matrices (protein-saccharide-water) exposed to treatments such as cooling and freeze-drying.

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