



Stabilizing effect of saccharides on bovine plasma protein: A calorimetric study

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ABSTRACT

Bovine plasma proteins provide the needed amino acids for the growth and development of an organism. With the purpose of preserving the native structure, related with the protein functional properties, the oligosaccharide inulin was used as protective agent and was compared with glucose and sucrose, during freeze-drying. In the present study, the thermal stability of protein was investigated as a function of type of saccharide in a concentration range of 5–15% (w/v), and at different pHs. The effect of these variables on phase transition, thermal stability and miscibility was assessed by differential scanning calorimetry (DSC) and scanning electron microscopy (SEM). The results of thermal protein properties (denaturation temperature and enthalpy), demonstrated that endothermic transition shifted to higher temperatures, being the stabilizing effect: inulin > glucose > sucrose. The thermal behavior suggests compatibility or interactions between the components of blends. In this way, the micrographs showed a homogeneous distribution of the different phases, corroborating the miscibility in the matrix. The unfolding process was irreversible and could be adequately described by a two-state model.

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1. Introduction

The use of bovine plasma proteins in food products is of great interest because of its functional and nutritional properties (Dávila, Parés, Cuvelier, & Relkin, 2007; Rodríguez Furlán, Pérez Padilla, & Campderrós, 2010a; Silva & Silvestre, 2003). In effect, previous studies demonstrated that plasma proteins showed good functional properties making them suitable for use in processed food for human consumption such as replacement for egg white in cakes (Myhara & Kruger, 1998), as fat replacers in ham pâté or in a hamburger (Rodríguez Furlán, Pérez Padilla, & Campderrós, 2010b; Viana, Silva, Delvivo, Bizzotto, & Silvestre, 2005). Moreover, the incorporation of bovine plasma proteins in food products may increase their nutritional value, because of the essential amino acid content and protein digestibility (Márquez, Bracho, Archile, Rangel, & Benítez, 2005; Oshodi, Beames, & Nakai, 1997; Viana et al., 2005). In addition, recent studies have demonstrated that the incorporation of bovine plasma hydrolysates improves the antioxidant properties of soybean and sunflower protein-based films (Salgado, Fernández, Drago, & Mauri, 2011).

For most of the applications in nutritional, technological, biological and pharmaceutical fields the preservation of the proteins in the

dry state is usually the best option, however during freeze-drying, a certain degree of deterioration may occur. During freeze-drying the protein is subjected to freezing and drying stress by which its activity can be lost (Allison, Chang, Randolph, & Carpenter, 1999). Therefore, the protein should be protected from conformational changes or denaturation. Previous studies have shown that during these stages, sugars yield two protectant effects: i) they replace the water that hydrates proteins and form with the protein hydrogen-bridge bonds, and ii) the protein is encapsulated within a vitreous structure avoiding its unfolding and thus preserving its conformation inhibiting crystallization that influence the kinetics of deteriorative reactions upon storage (Baeza & Pilosof, 2002; Buera, Schebor, & Elizalde, 2005; Carpenter, Prestrelski, & Arakawa, 1993; Claude & Ubbink, 2006; Santivarangkna, Higl, & Foerst, 2008).

Differential scanning calorimetry (DSC) is widely used to determine the thermodynamic of phase transitions in proteins, starch, etc. (Baeza & Pilosof, 2002; Krüger, Ferrero, & Zaritzky, 2003; Mali et al., 2003). Through the interpretation of DSC signal in terms of protein thermal denaturation it is possible to know the effect of type and concentration of saccharides in protein preservation. In particular there is little information on the influence of saccharides upon thermodynamic properties of bovine plasma proteins (Rodríguez Furlán, Lecot, Pérez Padilla, Campderrós, & Zaritzky, 2011). In this paper, the kinetics of bovine plasma protein stability in the amorphous inulin polysaccharide matrix was analyzed. The results were compared with a monosaccharide (glucose) and a disaccharide (sucrose). The

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thermal denaturation of freeze-dried bovine plasma proteins (BPP) by DSC, using different concentrations of the three protective agents, different scan rates, protein concentration, pH and the thermodynamic compatibility of the different matrix components, was determined to obtain more information about the stability of bovine plasma proteins, besides, a kinetic model that describes proteins denaturation was proposed. It is important to remark that the stability of bovine plasma proteins was studied employing inulin, which is a non-digestible carbohydrate widely used as an ingredient of functional food since it has beneficial effects on health as prebiotic: supply of dietary fiber, low calorie value, hypoglycemic, provision of a better bioavailability of calcium and magnesium, and enhance intestinal iron absorption (Wang, 2009; Yeung, Glahn, Welch, & Miller, 2006).

2. Materials and methods

2.1. Raw materials

Spray dried bovine blood plasma (Yerubá S.A., Esperanza, Argentina) has been used. The molecular weights of proteins were in the range of 15,000 to 80,000 Da. The proximate composition provided by the manufacturer was: $76 \pm 5\%$ proteins, $< 0.1\%$ fat, 10% ash, 4% water, 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 (Parafarm, Argentina) and inulin provided by Orafiti Chile S. A. obtained from chicory. The commercial inulin employed was mainly constituted, as was reported by the manufacturer, by linear chains of fructose, with a glucose terminal unit, has a molecular weight of 2400 g/mol and a polymerization grade (PG) of 12.

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

The feed solution was the bovine plasma which was dissolved in de-ionized water to a concentration of 3% w/v using a mixer at low speed to avoid the formation of vortex and to minimize the appearance of foam. The obtained solution was passed through a porous support (Viledon FO 2431D, Germany) to remove macroscopic aggregates. The feed (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. 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 operating conditions were the following: transmembrane pressure (ΔP) of 1.5 bar, flow rate of (2.9 ± 0.05) L/min and a temperature of 10 °C. A discontinuous diafiltration (DD) process was applied to removal salts and other contaminant of low molecular weight. For this operation the starting material 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 cleaning of the fouled membrane 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, pH = 12.5 ± 0.5)/water wash was applied to the membrane at (40 ± 2) °C and at a transmembrane pressure of 1×10^5 Pa. Furthermore, a cleaning step using NaClO (commercial grade) 300 ppm was carried out at the same temperature and pressure to ensure sanitation and

Table 1

Chemical average compositions of the bovine plasma proteins (BPP) freeze-dried with and without different saccharides (glucose, sucrose or inulin).

	BPP	BPP + Saccharide, 5%	BPP + Saccharide, 10%	BPP + Saccharide, 15%
Total average protein (g/100 g of product)	65.8 ± 0.6	54.8 ± 0.8	27.8 ± 0.4	14.2 ± 0.9
Average ash (g/100 g of product)	5.20 ± 0.13	4.90 ± 0.21	2.80 ± 0.13	1.65 ± 0.15
Average carbohydrate (g/100 g of product)	–	35 ± 2	64 ± 1	79 ± 1

cleaning. Measurements of normalized water permeability were performed in order to verify the recovery of flow through the membrane and the optimal performance during the separation process.

The bovine plasma protein (BPP) concentrate (concentration: 4% w/v), obtained by UF 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). The protein concentrates with the addition of the tested protective agents were placed on stainless steel trays and frozen in a freezer at -40 °C and freeze-dried using a lyophilizer (Rificor S.A., Argentina) at 1 bar of pressure for 48 h. The samples temperature was controlled by a temperature sensor. The denatured protein content was determined before and after the freeze-drying.

The chemical compositions of the freeze-dried samples with and without saccharides are given in Table 1. The components were expressed as average values, since there were no significant differences between different saccharides, at the same concentration.

2.3. Differential scanning calorimetry (DSC) measurements

Heat-induced conformational changes on freeze-dried bovine plasma protein concentrate (BPP concentrates) in the amorphous carbohydrate matrix at pH 8, 6 and 4 were monitored by DSC (Q100DTA Instrument, USA), at different heating rates of 2 and 5 °C/min in the temperature range 20–200 °C. The pH was adjusted using 0.1 N of NaOH and HCl. Protein concentrates (12.5 ± 2.5 mg) were weighed into aluminum DSC pans, hermetically sealed, and then loaded onto the DSC instrument at room temperature, using an empty pan as reference. The samples were scanned at 2 °C/min over the range 20 to 200 °C. The proteins in the calorimetric cell were reheated after cooling at 20 °C from the first run to estimate the reversibility of the thermally induced transitions. Measurements were carried out on three separate samples (replicates). The following parameters were calculated at least in triplicate: T_d (°C), defined as the denaturation temperature at maximum heat flow, and ΔH (J g^{-1}), the enthalpy change was 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 end of the transition temperature range (Akkose & Aktas, 2008; Cao et al., 2008; Dàvila et al., 2007).

2.4. Determination of native protein content

The native protein content was determined after isoelectric precipitation of denatured/aggregated protein (de Wit, 1990; Rodríguez Furlán et al., 2010a). Dispersions of 1% (w/v) protein concentrate were adjusted to pH 4.8 (the inferior value of the pI range of plasma proteins) 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 for 30 min at 5 °C. Protein concentration in the supernatants was determined by measuring absorption at 280 nm after appropriate dilution in a dissociating buffer (EDTA 50 mM, urea 8 M,

pH = 10) and reported as a percentage of the total protein concentration (Giroux & Britten, 2004). The percentage of native protein content of suspensions at pH 4.8 was calculated as the ratio between soluble protein (SP) and total protein (TP) contents after aggregation of denatured protein (Eq. 1).

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

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). The micrographs were obtained in high vacuum at 10 KeV. Powder samples were mounted on double-sided carbon adhesive tape on aluminum stubs and gold-coated and processed in a standard sputter.

2.6. Statistical analysis

The obtained data were statistically evaluated 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 (SAS, 1989) and using statistical GraphPad InStat Software (1998).

3. Theory

Protein unfolding can be reversible or irreversible. The two-state irreversible model, Eq. (2), includes both possibilities in two simple steps: (i) reversible unfolding of the native protein (N) to yield an unfolded state (D) and (ii) a change of the denatured protein into a final irreversible state (I) (Crevelde, Meijberg, Berendsen, & Pepermans, 2001; Idakieva, Parvanova, & Todinova, 2005)



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 a one-step process following first-order kinetics (Crevelde et al., 2001; Idakieva et al., 2005; Ramprakash et al., 2008), (Eq. 3).



where the first-order rate constant k can be identified with k_1 of Eq. (2). 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 (Crevelde et al., 2001).

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

The activation energy, E_a (J/g), from heat capacity curves of the thermal protein denaturation has been calculated by Eq. (4) according to Idakieva et al. (2005) and Sanchez Ruiz, Lopez Lacomba, Cortijo, and Mateo (1988):

$$k = A \exp\left(-\frac{E_a}{RT}\right) \quad (4)$$

where A is the pre-exponential factor and R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$). The units of the pre-exponential factor were identical to those of the rate constant and will vary depending on the order of the reaction. The rate constant, k (s^{-1}) was obtained at each

Table 2

Effect of saccharide concentration on the denaturation temperature of freeze-dried BPP concentrate (T_d of BPP without protective agent is 88.19 ± 1.87 °C).

Saccharide	Saccharide concentration (w/v)	T_d (°C)	ΔH (J g ⁻¹)
Glucose (MW = 180.1 g/mol)	5%	110.07 ± 1.22^a	0.84 ± 0.32^a
	10%	$107.27 \pm 0.85^{a,b}$	12.26 ± 0.92^b
	15%	104.91 ± 0.89^b	$3.77 \pm 0.98^{c,d}$
Sucrose (MW = 342.3 g/mol)	5%	132.78 ± 2.12^c	$5.08 \pm 0.98^{e,f}$
	10%	144.95 ± 2.34^d	22.40 ± 1.23
	15%	126.66 ± 1.54^e	7.01 ± 1.22^e
Inulin (MW = 2400 g/mol)	5%	143.81 ± 0.89^d	$2.97 \pm 0.55^{a,c,f}$
	10%	156.21 ± 1.12^f	12.22 ± 1.43^b
	15%	132.57 ± 1.34^c	$5.78 \pm 0.76^{e,d,f}$

Heating rate: 2 °C min⁻¹. pH = 8. Values represent the means \pm standard deviation. Values followed by different letters in the same column are significantly different from each other ($P < 0.05$).

temperature from the experimental data, according to the following equation:

$$k = \frac{v \tilde{C}_p}{\Delta H_{cal} - \Delta H} \quad (5)$$

where v refers to the heating rate (K s^{-1}), \tilde{C}_p is the heat capacity per gram ($\text{J g}^{-1} \text{ K}^{-1}$), ΔH_{cal} (J g^{-1}) is the total transition enthalpy, and ΔH (J g^{-1}) is the corresponding enthalpy at a certain temperature.

Crevelde et al. (2001), proposed the following model derived from considering that the thermal denaturation follows a two-state irreversible model. The model can be used to simulate the curves of heat capacity assuming the Arrhenius behavior for $k(T)$ in order to interpret the experimental data and to predict the behavior of the system.

$$\tilde{C}_p = \frac{k(T)\Delta H}{v} \exp\left(-\frac{1}{v} \int_{T_0}^T k(T) dT\right) \quad (6)$$

The equation relating the experimentally measured heat flow with the specific heat was as follows (Hewitt, Shires, & Bott, 1994):

$$\tilde{Q} = \tilde{C}_p \Delta T \quad (7)$$

where: $\tilde{Q} = Q/m$ (heat flow per mass unit) and $\Delta T = T_i - T_f$ with T_i and T_f : initial and final temperatures, respectively.

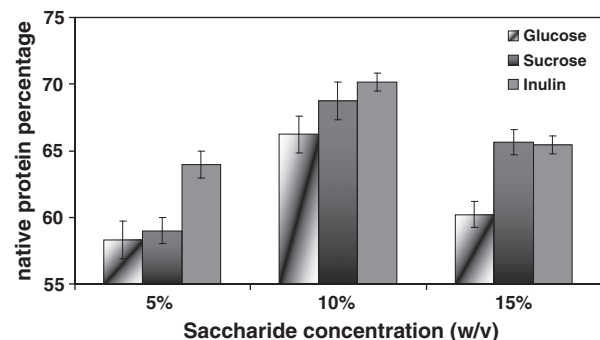


Fig. 1. Native protein percentage of freeze-dried BPP concentrate with different protective agents at different concentrations.

Table 3

Effect of pH and addition of saccharides on the denaturation temperature of BPP concentrate.

Saccharide	pH	T_d (°C)	ΔH (J g ⁻¹)
Glucose 10% (w/v)	8	107.27 ± 0.85 ^a	12.26 ± 0.82 ^a
	6	102.94 ± 1.33 ^b	34.74 ± 0.92 ^b
	4	101.74 ± 1.27 ^b	9.58 ± 0.98 ^{a,c}
Sucrose 10% (w/v)	8	144.95 ± 1.34 ^c	22.40 ± 0.97 ^d
	6	134.56 ± 2.16 ^d	43.15 ± 1.23 ^e
	4	107.67 ± 1.56 ^a	9.32 ± 0.72 ^c
Inulin 10% (w/v)	8	156.21 ± 1.12 ^e	12.22 ± 0.55 ^a
	6	152.98 ± 1.52 ^{e,f}	42.95 ± 1.45 ^e
	4	151.84 ± 1.89 ^f	9.35 ± 0.96 ^c

Heating rate: 2 °C min⁻¹. Values represent the means ± standard deviation. Values followed by different letters in the same column are significantly different from each other ($P < 0.05$).

4. Results

4.1. Effect of saccharide type and concentration on thermal denaturation of BPP

Table 2 shows DSC thermograms of BPP concentrate without protective agents and in different matrixes of glucose, sucrose and inulin at different concentrations. The T_d of the BPP concentrate (88.19 ± 1.87 °C), was similar to that obtained for blood plasma by Relkin (1996). The stabilizing effect of the saccharides was confirmed by the increase in T_d for all the samples with protein–polysaccharide matrix, indicating a higher thermal resistance. A similar behavior was achieved for the DSC study of whey protein concentrates with the addition of honey (Yamul & Lupano, 2003). Comparing among the saccharides at the same concentration, it can be observed that the higher the molecular weight of the carbohydrate, the higher was the T_d , thus inulin > sucrose > glucose. This result was in accord

to that obtained by Penco, Sartore, Bignotti, D'Antone, and Di Landro (2000), in multi-block copolymers. Considering, the concentration range assayed it was found that a carbohydrate concentration of 10% (w/v) produces optimal behavior in terms of T_d and ΔH (Table 2). It is known that the protect effect of non-covalent electrostatic complexes between protein and polysaccharides depends on the polysaccharide concentration (Allison et al., 1999; Rodríguez Furlán et al., 2010a). As oligosaccharide concentration was increased, its protectant effect increased too, due to there is more possibilities of forming hydrogen bonds with the protein (Allison et al., 1999). However, when concentrations were higher than 10% (w/v), a lower protection was obtained. This behavior can be explained considering that at high concentrations, the oligosaccharide starts to crystallize during freeze-drying, avoiding the formation of hydrogen bonds with the dry protein (Carpenter et al., 1993). This performance was confirmed by the determination of native proteins shown in Fig. 1 ($P < 0.05$), in which the higher concentration of native proteins was reached at a saccharide concentration of 10% for the three saccharides analyzed. This concentration was chosen for the following analysis.

4.2. Effect of pH on thermal denaturation of BPP

In Table 3 the parameters of the thermogram of BPP concentrate at different pH values are shown. The T_d values for each saccharide were higher at pH 8, indicating that BPP concentrate was more stable at higher pH. This result was in agreement with previous works in porcine blood plasma proteins and whey protein concentrate (Dávila et al., 2007; Yamul & Lupano, 2005). Comparing between different saccharides at the same concentration, it can be seen that inulin presents a higher T_d among all the pH range. The maximum ΔH values were observed at pH 6 indicating a higher amount of native protein at this pH. Similar ΔH values at pH = 6 were reported by Dávila et al. (2007). The destabilization observed at pH 4 (lower T_d and ΔH) could be explained by the approximation to the isoelectric point of

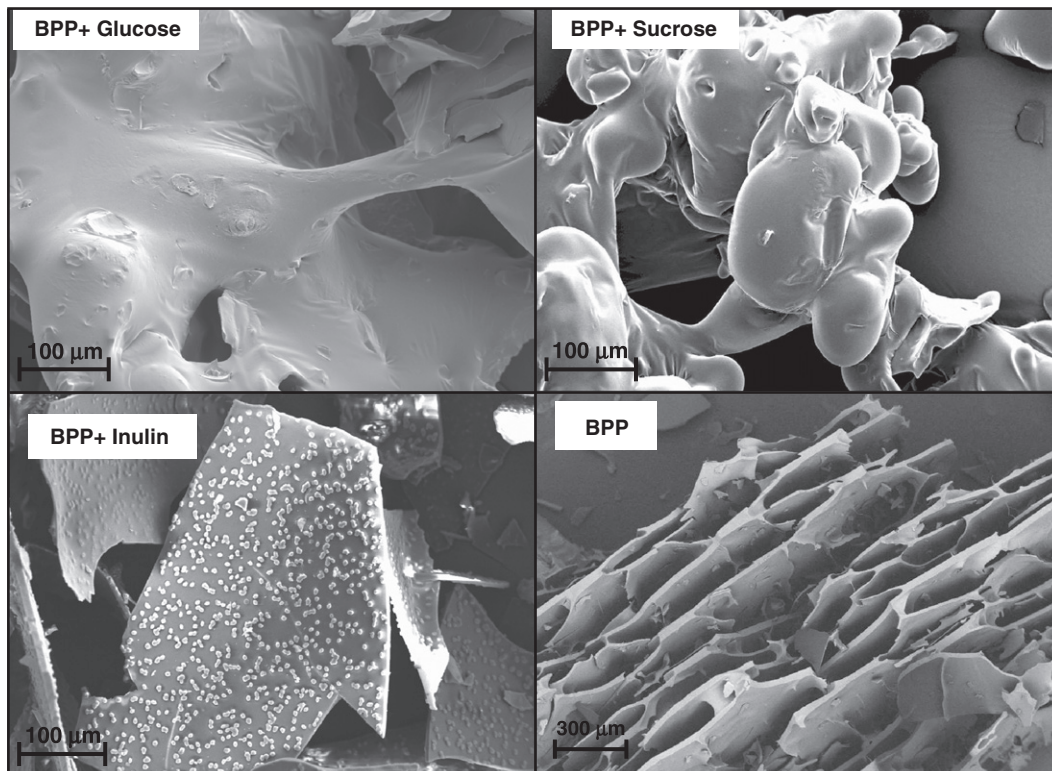


Fig. 2. Scanning electron micrographs of the freeze-dried product with and without different saccharides (magnification of 200× and 50×, respectively).

proteins (pI: 4.8–5.8), thus decreasing the electrical net charge and facilitating aggregation reactions.

4.3. Study of the morphology through SEM

Fig. 2 showed the SEM micrographics of BPP without protective agent and blends containing BPP and different saccharides at 10% (w/v) (glucose, sucrose and inulin). Phases homogeneously distributed can be seen in the blend samples, indicating miscibility of the component in the matrix. The size of the shapes was uniform in each sample, which was an attribute related with thermodynamic compatibility (Gallego, López, & Gartner, 2006). On the contrary, samples of freeze-dried plasma without saccharide showed a disrupted-like structure where the proteins presented a significantly affected microstructure (Rodríguez Furlán et al., 2010a,b). Furthermore, as was previously mentioned, there was a noticeable change in the transitions of the blends (higher T_d) with respect to the value of individual components. The changes in the T_d values can be attributed to greater miscibility of the mixture components confirming what was observed in the micrographs. This statement is in accord with the concept of miscibility, which is represented by the thermal behavior variation with respect to the individual materials (Mousavioun, Doherty, & George, 2010).

4.4. Effect of protein concentration on thermal denaturation of BPP

It is important to check if the calorimetric profiles for bovine plasma proteins are protein concentration-dependent, since the dependence on the position of the transition temperature T_d gives information about the changes in the molecularity occurring during thermal denaturation process (Michnik, Drzazga, Kluczevska, & Michalik, 2005). Different protein concentrations were investigated, from 16 to 80% (w/v), obtaining values of T_d and ΔH from 69–89 °C and 4.8–568 J/g, respectively. Therefore, protein concentration substantially affected thermal denaturation of BPP. The plot of the logarithm of protein concentration versus $1/T_d$ is linear with a negative slope, which indicates that the bovine plasma proteins experimented dissociation on denaturation. The ΔH (area under the peak) also increased as BPP concentration increased. Similar results were found for Cao et al. (2008) for BSA protein.

4.5. Effect of scanning rate on thermal denaturation of BPP

The experiments carried out for the different saccharides at 10% (w/v) at different scans speed (2 °C/min and 5 °C/min), showed that the scanning rate affected the transition temperature and enthalpy. T_d values of saccharides increasing 5 ± 2 °C in all the samples with increasing scanning rate, as was observed by Kavitha, Bobbili, and Swamy (2010), Schubring (1999), Zamorano et al. (2004). Furthermore, the ΔH decreased, approximately 10%, with increasing scanning rate, which was in agreement with the findings of Guzzi, Sportelli, Sato, Cannistraro, and Dennison (2008) and Vermeer and Norde (2000). In this way it was proved that the system was scanning-rate dependent and so the thermal denaturation process was under kinetic control (Cao et al., 2008; Creveld et al., 2001).

4.6. Study of irreversibility of the thermal denaturation of BPP

Thermal unfolding of BPP concentrate is a complex process. 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 2). According to the method proposed by Idakieva et al. (2005) and Michnik et al. (2005), a multiple DSC reheating scan was carried out. For glucose, in the first run, the heating was stopped at 75 °C, no thermal effect was observed in the area of the peak in the reheating experiment. However, if the heating in the rescanning was stopped over the second maximum, the

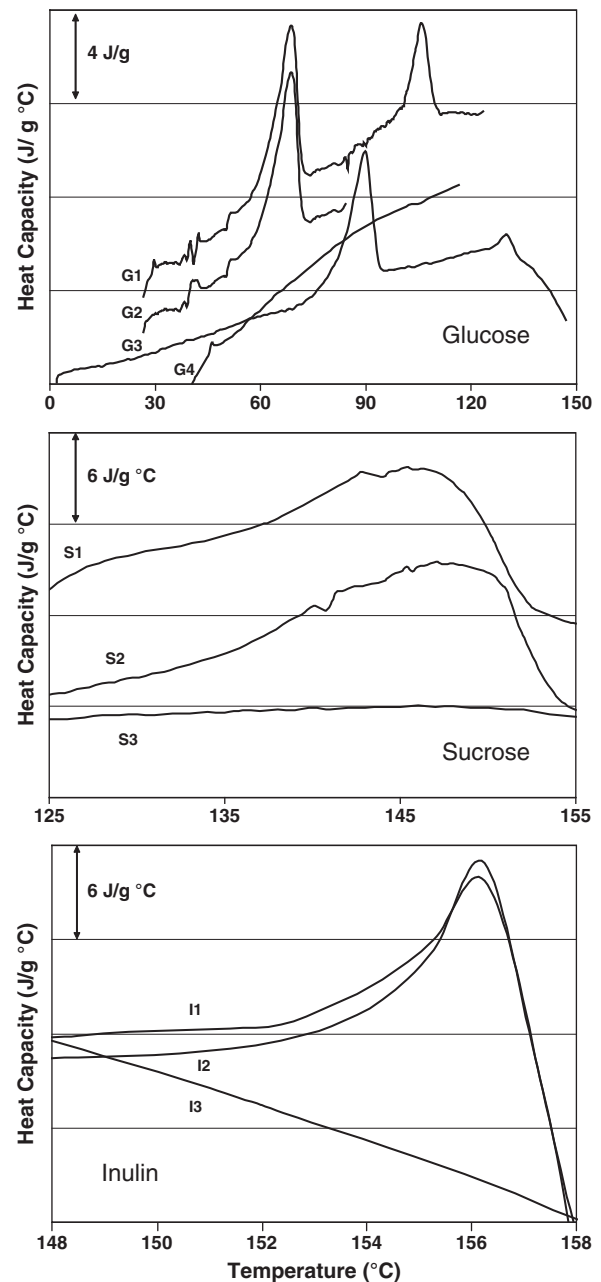


Fig. 3. DSC thermograms of freeze-dried BPP concentrate with glucose, sucrose and inulin at 10% (w/v). DSC full scan: G1; S1; I1 at 120 °C, 155 °C and 158 °C, respectively. DSC rescans: G2, G3, G4; S2, S3; I2, I3, represent thermograms from repeated heating and subsequent cooling.

endothermic peak of T_d disappeared completely. For inulin and sucrose the first scan was stopped at 86 °C (temperature below the T_d for both saccharides). For sucrose and inulin, reheating did not show any difference in the main peak. When the heating was stopped at the maximum point (T_d) for each saccharide and in the rescanning no transition could be seen. The results are shown in Fig. 3 for all the saccharides. Therefore, the endothermic peak of T_d disappeared completely upon rescanning the sample; furthermore, as was previously described, the thermograms were scanning-rate dependent, suggesting both results that it was an irreversible event. A similar behavior was also found for BSA (Cao et al., 2008), porcine blood plasma proteins (Dávila et al., 2007) and whey protein in an amorphous carbohydrate matrix (Sun, Davidson, & Chan, 1998). The plasma proteins

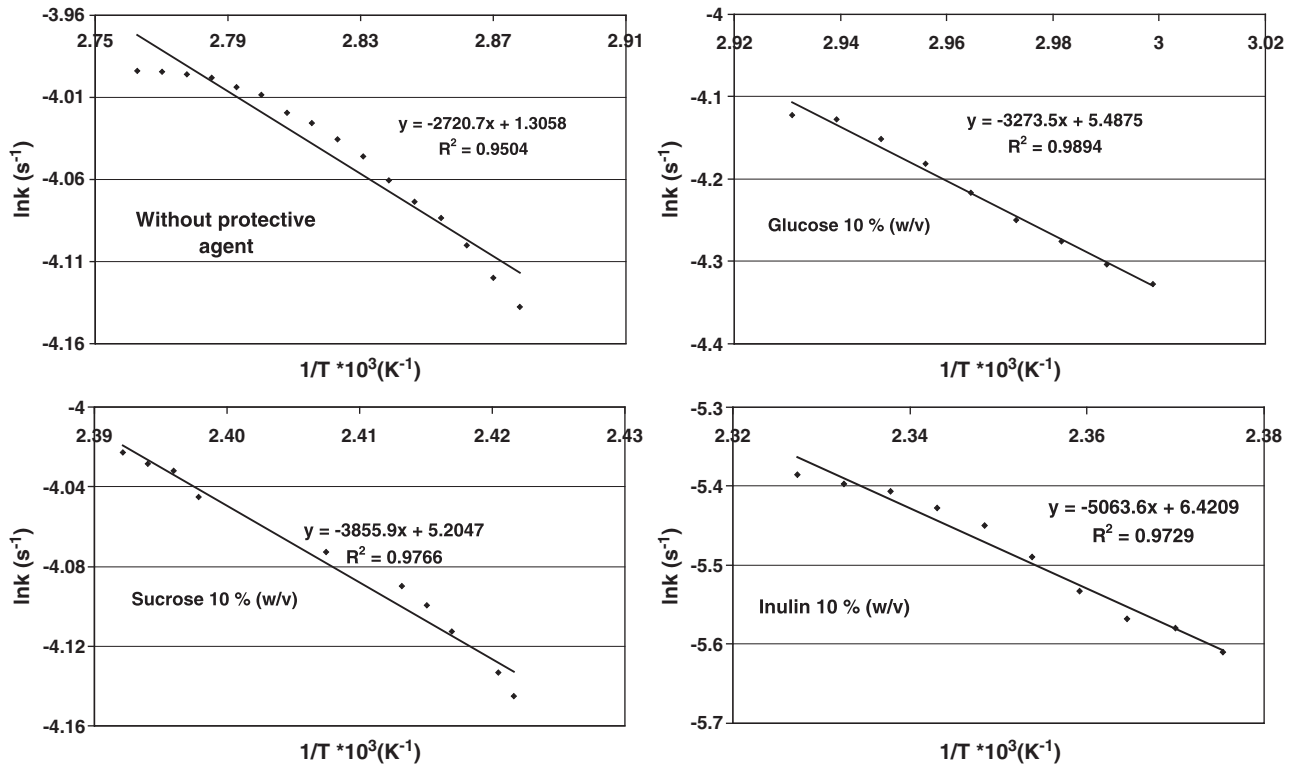


Fig. 4. Effect of protective agent addition on activation energy of freeze-dried BPP concentrate aggregation.

denaturation has been explained considering the occurrence of “side” processes such as aggregation. Due to denaturation, hydrophobic interaction can occur, and exposed thiol groups can form disulfide bonds, which result in an irreversible behavior (Cao et al., 2008).

Fig. 4 represents the Arrhenius plot of the kinetic parameter $\ln k$ versus T^{-1} , the k parameter has been obtained from Eq. (4). DSC data were used to represent the thermal denaturation of BPP in terms of the two-state kinetic model and allowed the determination of the activation energy obtained from the slopes of the plots. The values were: $10,443 \text{ J mol}^{-1}$ for BPP without protective agent; $27,216 \text{ J mol}^{-1}$, $32,058 \text{ J mol}^{-1}$ and $42,099 \text{ J mol}^{-1}$ for BPP with glucose 10% (w/v), sucrose 10% (w/v) and inulin 10% (w/v), respectively. The results showed that the samples with protective agents had higher values of activation energy than BPP alone. Furthermore, the higher the saccharide molecular weights, the higher were the activation energies. This means that the amorphous systems inhibit crystallization and influence the kinetics of deteriorative reactions, obtaining a higher stabilization upon storage (Buera et al., 2005). The larger E_a value of inulin would be due to the fact that this oligosaccharide has a lower tendency to crystallization than disaccharides and monosaccharides, being a better stabilizer (Hinrichs, Sanders, De Smedt, Demeester, & Frijlink, 2005; Rodríguez Furlán et al., 2010a).

4.7. Irreversible denaturation behavior of BPP

The behavior of the protein–saccharide mixture as a function of temperature, can be predicted from the theoretical values of \tilde{C}_p as a function of T from Eq. (6), where $k(T)$ was obtained from Eq. (5), ΔH and v were experimental conditions.

Fig. 5 shows, together with the experimental thermogram of BPP concentrate with the different saccharides, the theoretical denaturation curve. Non-significant differences between T_d from the theoretical and the experimental curves were observed, presenting similar shapes, in agreement with the proposed two-state irreversible

model. The shape of the curve may be explained by the cooperative process (stabilizing effect of saccharide) that takes place by the binding of BPP at extended hydrophobic patches, which were present at the surface of the unfolded molecules (Crevelde et al., 2001).

5. Conclusions

The ultrafiltration–discontinuous diafiltration step (UF–DD) allows concentrating protein, eliminating insoluble macroscopic components and reducing the saline content.

The DSC thermograms demonstrate that the carbohydrate matrix has a stabilizing and protective effect on protein structure during freeze-drying, increasing the protein denaturation temperature (T_d) and enthalpy (ΔH), with an optimal saccharide concentration of 10% (w/v) and a pH ranging between 6 and 8. 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 rescans experiments so it was concluded that the protein unfolding was irreversible and was adequately interpreted by the theoretical two-state irreversible model employed.

The result of this study clearly illustrates that the polysaccharide inulin exhibit better stabilizing properties than the disaccharide sucrose and the monosaccharide glucose for bovine plasma proteins. This finding is relevant considering that inulin is a soluble fiber, categorized as a prebiotic, being a valuable functional ingredient for food formulations from bovine plasma proteins.

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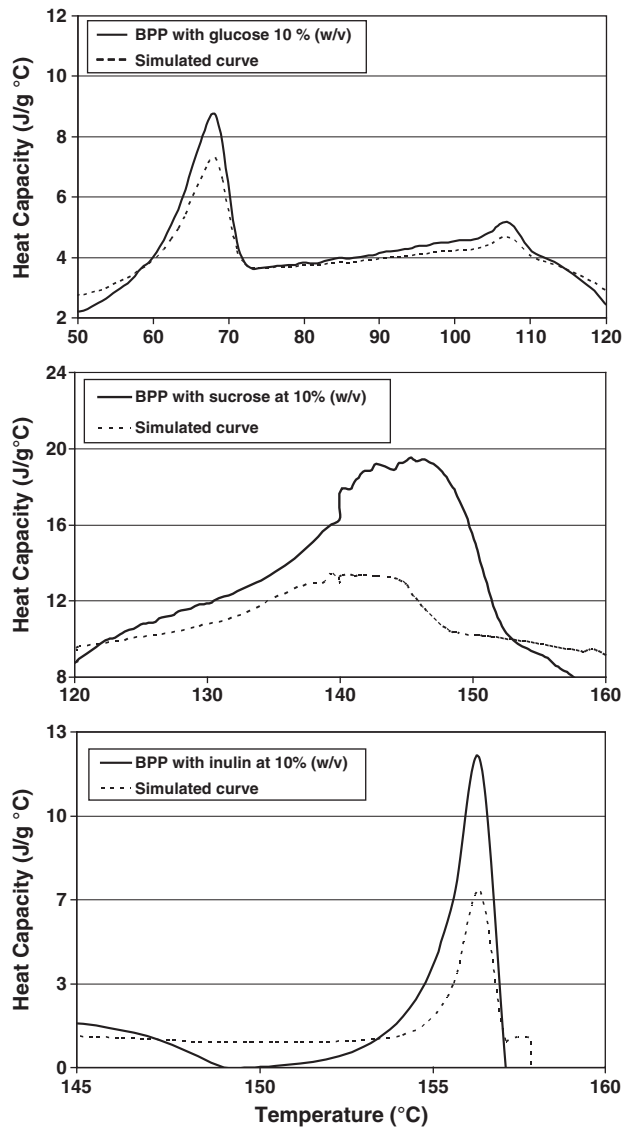


Fig. 5. DSC denaturation experimental transitions for freeze-dried BPP concentrate with different saccharides at 10% (w/v) (full line). A simulated curve assuming a two-state model is included (dashed line). The scan rate is $2^{\circ}\text{C min}^{-1}$.

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