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Inulin like lyoprotectant of bovine plasma proteins concentrated by ultrafiltration

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ABSTRACT

The growth of world demand for protein-enriched products generates a great interest in the search of new protein sources of higher nutritional value and, therefore, in the use of cutting edge technologies to achieve that goal. In this work, technologies of ultrafiltration and freeze-drying to process and obtain a protein concentrate from bovine plasma proteins are employed. The effectiveness of inulin as protectant agent to prevent protein denaturation was evaluated during freeze-drying and storage. The shelf life time of the product with the oligosaccharide was estimated to analyze its stabilizing power. The results showed that when 10% w/v inulin was employed as lyoprotectant the protein denaturation was significantly reduced with a shelf life of 14 months, stored at 2 °C. The proteins concentrate combines improved functional properties due to reduction of protein denaturation, indicating that it could be very interesting as food ingredient.

1. Introduction

The growth of world population generates an increasing demand for food which is not yet satisfied in many regions of the Planet. For this reason the development of new sources of proteins and the optimization of the existing ones are issues of great interest and study. The bovine plasma is obtained from blood which is the principal highly contaminating organic pollutant discharged in wastewaters of the meat industry, nevertheless it contains animal proteins with excellent nutritional properties. The food industry could use proteins from blood as additives in dietetic or other product, such as: dairy products, drinks, bread, beverages, ice-creams, soufflés, cake, meat products, etc. (Del Hoyo, Rendueles, & Díaz, 2008). One of the factors with a greater influence in the decrease of nutritional and functional value of protein concentrates is protein denaturation which is a total or partial loss of structure levels higher than the primary. This fact is principally due to the rupture of weak hydrogen bonds and Van der Waals interactions (Cheftel, Cuq, & Lorient, 1989).

The way of conducted proteins processing and storage considerably affects its properties. A conventional method used for the concentration and demineralization of bovine plasma is the ultrafiltration (UF) (Dailloux, Djelveh, Peyron, & Oulion, 2002; Del Hoyo, Moure, Rendueles, & Díaz, 2007; Del Hoyo et al., 2008; Fernández, Rodríguez, & Pérez Padilla, 1999; Roodink & Niewold, 2003). It is more efficient than traditional methods because it is a one-stage model with low energy consumption and without generating contaminating by-products (Belhocine, Grib, Abdessmed, Comeau, & Mameri, 1998). The UF systems operate at low temperatures allowing the separation and concentration of heat-sensitive biological preparations, obtaining a higher quality product (Boye et al., in press; Fernández, Menéndez, Mucciarelli, & Pérez Padilla, 2007; Noordman, Ketelaar, Donkers, & Wesselingh, 2002; Rinaldoni, Tarazaga, Campderrós, & Pérez Padilla, 2009).

The protein concentrate can be dried by freeze-drying, which simplifies aseptic handling and enhances stability of dry powder, without excessive heating of the product (Fellows, 1994). However, 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 exert 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 it is unfolding and thus preserving its conformation (Buera, Schebor, & Elizalde, 2005; Carpenter, Prestrelski, & Arakawa, 1993; Minson, Fennema, & Amundson, 2006).

The inulin has proved to be an excellent protect of therapeutical proteins and viruses over the drying and storage processes (Hinrichs, Prinsen, & Frijlink, 2001; Hinrichs, Sanders, De Smedt, Demeester, & Frijlink, 2005). Also it has been assayed as protectant to maintain the viability of probiotic organism during freeze-drying of yoghurt (Capela, Hay, & Shah, 2006). This oligosaccharide is an effective lyoprotect since it is a stabilizing agent with a high glass transition temperature (T_g), lower number of reducing groups and a low rate of crystallization which extends the product shelf life

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(Jonge, Amorij, & Hinrinchs, 2007). This compound is a non-digestible carbohydrate present in many vegetables, fruits and cereals. It is 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 (Lingyun et al., 2007; Wang, 2009; Yeung, Glahn, Welch, & Miller, 2006). Besides, there are promising evidences of its performance in the regulation of lipid parameter, reduction of cancer risk, reinforcement of the immune response and protection against bowel disorders.

In this work the effectiveness of inulin as lyoprotectant of bovine plasma proteins was investigated after processing by ultrafiltration and freeze-drying, and during storage. The results obtained with inulin were compared with those obtained for disaccharides (sucrose) and monosaccharides (glucose). During the freeze-drying process, temperature operative conditions were studied in order to optimize and reduce energy expense.

2. Materials and methods

2.1. Raw materials

Spray dried bovine plasma (Yerubá S.A., Argentine) has been used. The molecular weights of proteins are in the range of 15,000–80,000 Da. The composition is given in Table 1.

Inulin provided by Orafti Chile S. A. and obtained from chicory has been used. 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. They are used in a wide variety of foodstuffs as: thickener, emulsifier, gelling agent, sugar and fat substitutes, humectants, depressing of the freezing point, among others (Hennelly, Dunne, O'Sullivan, & O'Riordan, 2006; Rosell, Santos, & Collar, 2009; Tseng & Xiong, 2009).

2.2. Determination of denatured protein contain

The soluble protein content was determined after isoelectric precipitation of denatured/aggregated whey protein (de Wit, 1981, 1990; Meza, Verdini, & Rubiolo, 2009; Verheul, Roefs, & de Kruif, 1998). Solutions of 1% (w/v) protein concentrate were adjusted to pH 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 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 percentage of the total protein concentration (Giroux & Britten, 2004). Insoluble protein content of suspensions at pH 4.8 was defined as the difference between total protein (*TP*) and soluble protein (*SP*) contents and was used to estimate the extent of denaturation/aggregation of whey protein (Anandharamakrishnan, Rielly, & Stapley, 2008). The percentage of denatured protein (*DP*) content was calculated with the following equation (Morr, 1990):

$$DP = \frac{(TP - SP)}{TP} 100 \quad (1)$$

Table 1
Bovine plasma composition (Yerubá S.A.).

Component	Content (%)
Protein	76 ± 5
Ash	10
Humidity	4
Low molecular weight compounds	1

The measures have been done in the raw material and in the protein concentrates with and without protectant agent in all the stages of the procedure.

2.3. Ash determination

The ash content measurement was carried out in a muffler furnace (Indef 132, Argentine) using a controlled temperature program to gradually reach 520 °C. The salt reduction percentage was determined by the difference between the initial and final weight of the samples.

2.4. Ultrafiltration–diafiltration stage

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 F 02431 D, 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, Argentine). This procedure reduces the amount of bacteria and spores and acts cold pasteurization. Moreover the MF stage protects the UF membrane from fouling. The ultrafiltration (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 ultra filtrated 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 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 and the optimal performance during the separation process.

2.5. Freeze-drying stage

The bovine plasma protein concentrate (concentration: 4% w/v), obtained by UF was fractioned. A fraction as witness sample was reserved and the protect solution (inulin) was added to the rest, in concentrations of 1%, 4%, 10% and 15% (w/v). The protein concentrates were placed on stainless steel trays and frozen in a freezer at −20 and −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 contain was determined before and after the freeze-drying.

2.6. Functional characterization of the protein concentrates

Functional properties of food proteins are important in food processing and food product formulation. Some of these properties are solubility, emulsifying capacity and foam capacity.

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The method of Lee, Morr, and Ha (1992) with several modifications was used to determine the protein solubility at room temperature (22 ± 1) C. 1% (w/v) solution of each lyophilized protein concentrates in 0.01 M phosphate buffer (pH adjusted to between 3 and 9 with either 1 N HCl or 1 N NaOH, respectively) was prepared and stirred for 5 min. Then it was centrifuged in a refrigerated ultracentrifuge (Beckman J2-HS, USA) at 20,000 rpm for 30 min at 5 °C. The protein content of the supernatant was determined by absorption spectroscopy (Double beam Shimadzu Spectrophotometer, USA) at 280 nm, bovine serum albumin (BSA) was used as standard. The protein solubility was calculated as the percentage of protein in supernatant divided by the percentage of total protein in the original solution. The 0.01 M phosphate buffer was used instead of sodium chloride solution in preparing the standards because phosphate buffer was used as extraction medium (Ee, Rehman, Agboola, & Zhao, 2009). The experiences were carried out in triplicate.

The emulsifying capacity (EC) was performed as described by Yu, Ahmedna, and Goktepe (2007) with minor modifications. One gram of each protein concentrate was mixed with 200 mL of deionized water for 2 min using a mixer at high speed before slowly addition of 500 mL of vegetal oil under continuous blending. Blending was stopped every 2 min to check for emulsion breakage. When a clear emulsion breakage was observed, the total volume of oil added was recorded and used to calculate EC as volume (mL) of oil emulsified per gram of protein concentrate.

The foaming capacity (FC) of the protein concentrates was investigated using a simple technique as described by several workers (Chove, Grandison, & Lewis, 2007; Coffman & Garcia, 1977; Makri, Papalamprou, & Doxastakis, 2005). 1% (w/w) protein solutions were prepared in de-ionized water and adjusted to pH 7.4 with 1.0 N NaOH and HCl. Triplicate samples of the dispersions (V_I = 100 mL) were blended in a graduated measuring jug using a mixer for 30 s (1000 rpm). The final solution volume including the produced foam (V_F) was immediately reported. FC was calculated as the ratio of V_F to V_I. The samples were reserved at room temperature (22 ± 1) °C and the foam volumes read at 1 h. The foaming stability (FS) was calculated as the percent ratio between at 1 h to that initial (Marco & Rosell, 2008; Yusuf, Ayedun, & Sanni, 2008).

2.7. Shelf life time study (kinetics of thermodegradation)

As a general rule, food stability is the parameter that defines the product commercial value. Therefore, it is important to study the mechanisms and general kinetic principles of degradation of the less stable components which determine the product quality. This work analyzes the kinetics of the native proteins in the protein decrease concentrate and the shelf life time prediction under proper storage and distribution conditions.

The analytic approach to calculate and predict the quality deterioration of food involves a kinetic/mathematic model. The experimental procedures to determine the destruction kinetics of food components have been widely investigated and there are a number of methodologies to predict the loss of nutrients (Dattatreya, Etzel, & Rankin, 2007; Hough, Calle, Serrat, & Curia, 2007). Kinetic parameters are sensitive to factors such as food composition and the process characteristics.

The following procedure was used: 10 g freeze-dried samples were placed in airtight bags with toothed zip fastener (Ziploc, S. C. Johnson & Son), protecting the product from contact with oxygen and water vapor. Samples were preserved from light exposure and then, stored in ovens at constant temperatures of 25, 40 and 60 °C (with a variation of ± 2 °C), for 60 days. They were tested in duplicate on a weekly basis.

2.8. Kinetic degradation model

The kinetic degradation model employed to predict the loss of native protein in the concentrate has been provided by Labuza and Riboh (1982), by the following general equation:

$$-\frac{d[F]}{dt} = k[F]^n \tag{2}$$

where: $[F]$ = quality factor concentration; k = degradation velocity constant; n = reaction order.

Integrating Eq. (2), for $n = 1$:

$$-\ln\left(\frac{[F]_t}{[F]_0}\right) = -kt \tag{3}$$

where the subscripts *0* and *t* were at initial time and at time (t), after the degradation reaction, respectively.

The relationship between the reaction constant and the temperature is quantified by the Arrhenius equation (Labuza & Riboh, 1982; Robertson, 1993):

$$k = k_0 e^{\left(-\frac{AE}{RT}\right)} \tag{4}$$

where AE is the activity energy of the reaction (cal/mol), R the gases universal constant (1.987 cal/mol K); T the absolute temperature (K) and k_0 is the pre-exponential constant or frequency factor (1 min^{-1}).

2.9. Statistical analysis

The obtained data were statistically evaluated by the Tukey–Kramer multiple comparison test in the cases where two or more comparisons were considered. Otherwise, the *T*-test was used, assuming that a $P < 0.05$ was statistically significant (SAS USER GUIDE: Statistic. Versión, 1989).

2.10. Scanning electron microscopy

The microstructure of freeze-dried plasma concentrates with and without inulin addition was analyzed by scanning electron microscopy (SEM) using an LEO1450VP equipment (Zeiss, Germany). Powder samples were mounted on metal stubs and gold-coated to obtain surface micrographs.

3. Results and discussion

3.1. Effect of ultrafiltration–diafiltration stage

The ultrafiltration shows many advantages over traditional methods. It is a non-thermal process, does not require chemical compounds to be added and is economically more viable than other treatments such as vacuum evaporation used for concentration of animal blood (Fernando, 1981; Noordman et al., 2002; Torres, Marín, Ramos, & Soriano, 2002).

The UF–DD step allows concentrating protein, eliminating insoluble macroscopic components, and reducing the saline content. In this sense, the raw material has an ash content of 9.9 ± 0.08% (w/w), while the concentrate by UF–DD has 5.2 ± 0.13% (w/w), so the mineral content has been reduced a 47.5 ± 0.5%, being more adequate for food formulations. This result is in agreement with those reported by Del Hoyo et al. (2007). Furthermore, the plasma protein had an initial denatured protein of 30.09 ± 0.95% (w/v) and after the UF–DD process, the concentrate had 27.17 ± 0.74% (w/v), resulting lees affected than protein treated with others procedures as high pressure processing employed in red blood cell fraction (Toldrà et al., 2008).

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3.2. Effect of inulin on protein protection during the freeze-drying

Fig. 1 shows inulin protectant effect at concentrations of 1%, 4%, 10% and 15% (w/v). It can be seen that 10% (w/v) was the best concentration in comparison with the sample without protect ($P < 0.001$) with a denatured protein percentage of 29.85 ± 0.65%. No statistically significant difference was observed between the protein concentrate with the addition of inulin at 1% (w/v) (39.41 ± 1.20) and without protector (39.25 ± 2.83) ($P > 0.05$). However, in the case of inulin addition at 4% (w/v) (34.06 ± 0.26) ($P < 0.01$) and 15% (w/v) (33.38 ± 0.68) ($P < 0.05$), there was a statistically significant difference in relation to the sample without protector. Comparing inulin at 4% and 15% (w/v), there was not a statistically significant difference ($P > 0.05$).

The functional structure of a protein in solution was determined by electrostatic forces, hydrogen bonds, Van der Waals interactions and hydrophobic interactions. All these interactions were influenced by water, becoming essential for the functional unfolding of most of the proteins (Wolkers, Van Kilsdonk, & Hoeskstra, 1998). 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, and a β-laminar structure is obtained (Allison et al., 1999; Carpenter et al., 1993; Hinrichs et al., 2001, 2005, 2006). 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 (Allison et al., 1999; Buera et al., 2005; Carpenter et al., 1993). 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 (Allison et al., 1999; Buera et al., 2005; Carpenter et al., 1993; Murray & Liang, 1999).

As oligosaccharide concentration was increased, its protectant effect increased too. This is due to the fact that there are 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 may be due to the fact 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).

In Fig. 2, the lyoprotectant effect of sucrose, glucose and inulin at 10% (w/v) are compared. No statistically significant difference was observed with respect to the protection exerted by glucose (33.48 ± 1.40), sucrose (30.23 ± 2.30) and inulin at 10% (w/v) (29.85 ± 0.65) (w/v) ($P > 0.05$). However, when comparing them with the solution without protector ($P < 0.01$), there was an observable difference. Therefore, inulin has similar lyoprotectant characteristics to monosaccharides and disaccharides.

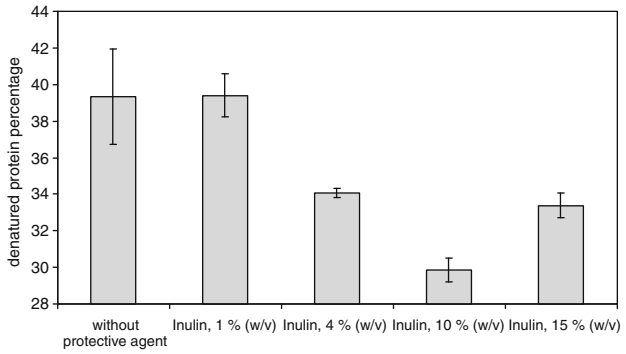

Fig. 1. Overall denatured protein content: effect of inulin at different concentrations on bovine plasma protein denaturation. The error bars represent standard deviations.

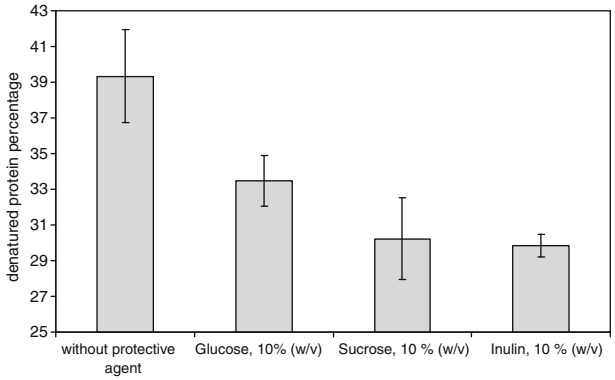

Fig. 2. Overall denatured protein content: comparison among different protectant agent *s*: mono and disaccharides with inulin during freeze-drying process (concentration, 10% (w/v), $T = -40$ °C). The error bars represent standard deviations.

Freeze-drying is a relatively expensive process, because of the high energy demands lead to high energy costs. Depending on the good results obtained for inulin as lyoprotectant, a freeze- drying was carried out to major temperature to evaluate its behavior. The protein concentrate with 10% (w/v) inulin was frozen at −20 and −40 °C. Fig. 3 shows the protectant effect of inulin. There was no statistically significant difference between freeze-dried bovine plasma at −20 and −40 °C ($P > 0.05$). Therefore, a product of similar quality was obtained (the same denatured protein percentage) with a significant decrease of energy consumption since it could be frozen at a higher temperature, −20 °C, due to a high value of inulin T_g. The low operating temperature of the process leads to minimal damage of the heat-sensitive products as proteins. By freeze-drying the protein concentrate without the addition of inuline at −20 °C, yields a gummy product, due to the low value of T_g of the solution.

3.3. Effect of inulin on protein functional properties

From a practical point of view, data about solubility characteristics are quite helpful in determining the optimal conditions of proteins extraction and purification, as well as the separation of protein fractions. Under different conditions, solubility is also a good indicator of the potential applications of proteins and influences other functional properties (Cheftel et al., 1989; Yu et al., 2007).

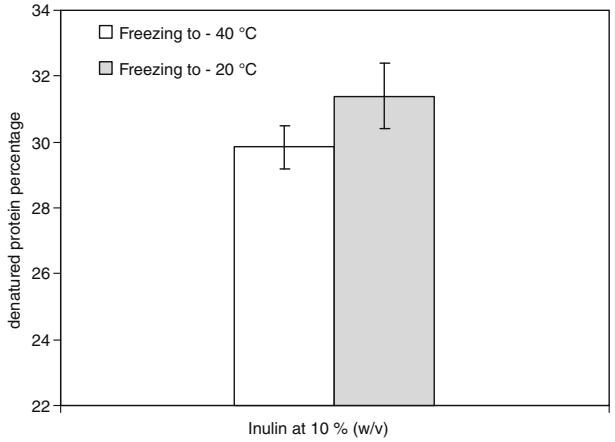

Fig. 3. Overall denatured protein content: denatured protein using inulin at 10% (w/v) at different freezing temperatures. The error bars represent standard deviations.

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As show in Fig. 4 the plasma bovine proteins show a maximum solubility at pH 7.5 ± 0.3 and a decrease between pH 4 and 6. This agrees the range within their proteins isoelectric point can be found (pH 4.6–5.2). This behavior is in accord with the results reported by Del Hoyo et al. (2008). The plasma concentrate without the protectant agent has lower solubility than the concentrate with inuline. This can be explained considering that the proteins solubility is inversely proportional to the concentration of denatured proteins (Cheftel et al., 1989; Toldrà et al., 2008). Moreover, the presence of the polysaccharide, which in solution acts as charges that adsorb on the protein surface diminishing the interaction protein–protein, and hereby the protein tends to remain in solution (Cheftel et al., 1989; Yusuf et al., 2008).

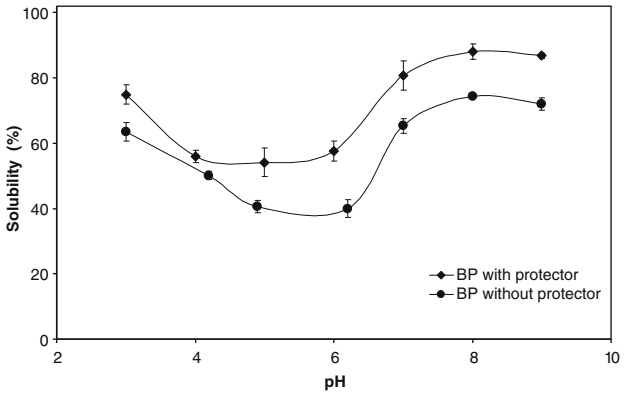

Fig. 4. Solubility of protein concentrates at different pH, ($T = 22 \pm 1$ °C). The error bars represent standard deviations.

Table 2
Characteristics of the freeze-dried protein concentrates.

Properties	Concentrate without protector	Concentrate with inulin
EC (ml of oil/g of product)	289 ± 8	500 ± 9
FC (ml/ml)	1.83 ± 0.08	1.63 ± 0.13
FS (%)	22 ± 2	53 ± 3

Food emulsions are thermodynamically unstable mixtures of immiscible liquids (water and oil). The formation and stability of emulsion is very important in food systems such as salad dressings, ice cream, confectionary or meat products, etc. (Rodríguez Patino, Carrera Sánchez, & Rodríguez Niño, 2008; Yu et al., 2007). As shown in Table 2 the protein concentrate with inulin presents a higher emulsion capacity, this could be due to the presence of the polysaccharide, decrease the surface tension and thus the free energy of the system, and impart the desired kinetic stability to dispersions (emulsions or foam), (Rodríguez Patino et al., 2008).

In the formation of foam the water molecules surround air droplets, and air is the non-polar phase. Theoretically, the amphipatic character of protein makes them the good foaming agents that work at air–water interface to prevent bubble coalescence (Yu et al., 2007). Data in Table 2 show that the foam capacity is similar for both protein concentrates, while the stability is higher for plasma concentrate without the protectant agent, this behavior may be explaining considering that protein-polysaccharide complexes enhance emulsion stability due to steric repulsion effects.

It can be seen in that the addition of saccharose impairs foamability, but improves foam stability. This fact was reported by Sikorski (2007), who explained that the positive effect of sugars on foam stability is due to increased bulk-phase viscosity, which reduces the rate of drainage of the lamella fluid. The depression in foam overrun is mainly due to enhanced stability of protein structure

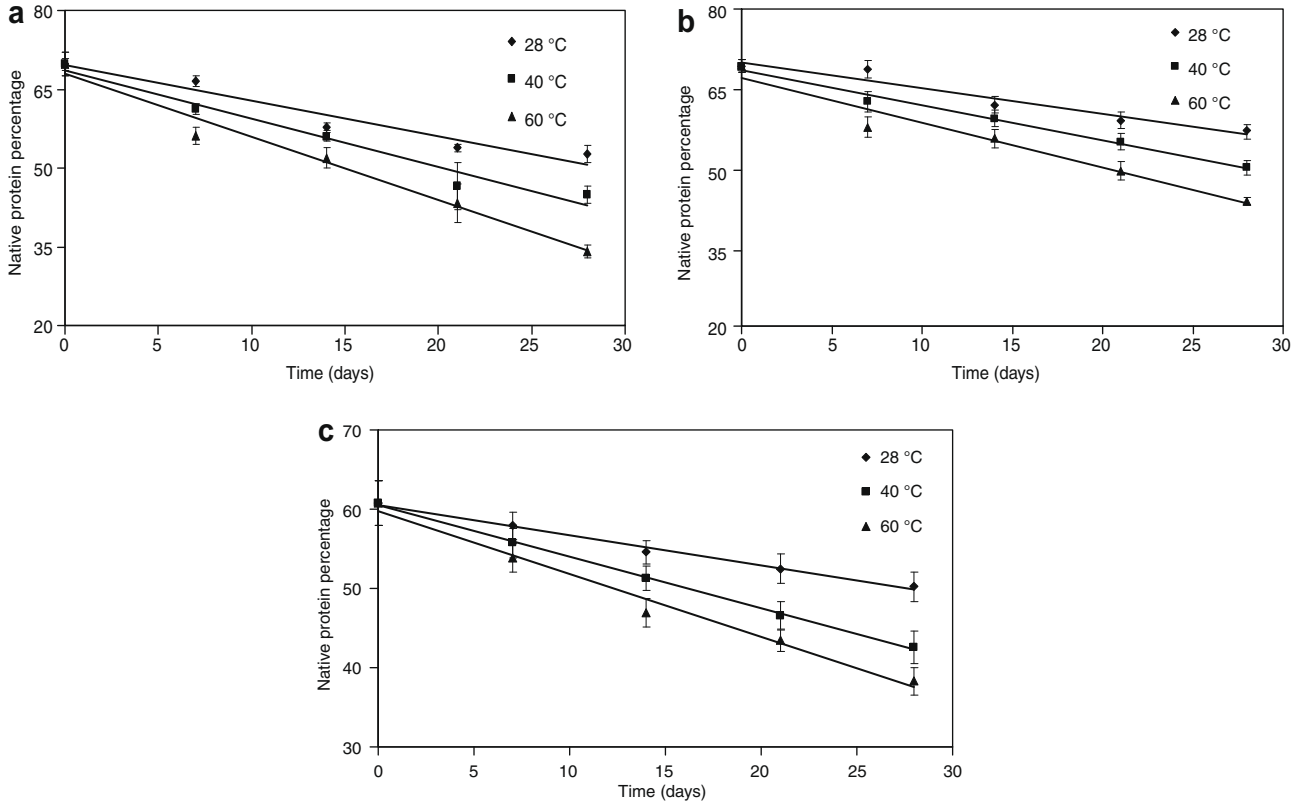

Fig. 5. Native protein content at different storage temperatures: (a) protected with sucrose at 10% (w/v); (b) protected with inulin at 10% (w/v); (c) without protectant agent .

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in sugar solutions. Because of this, the protein molecule is less able to unfold upon adsorption at the interface. This decreases the ability of the protein to produce large interfacial areas and large foam volume during whipping. The effect has been reported by several authors as Fidantsi and Doxastakis (2001) for amaranth seed protein isolates; Rodríguez Patino et al. (2008) in foam formulations, Ee et al. (2009) in wattle seed; between others. Comparing with blood proteins processed by high pressure (Toldrà et al., 2008), the plasma proteins with inulin have better foaming capacity and better foam stability.

3.4. Effect of inulin on shelf life time

In Fig. 5, the variation in time of the quality factor indicator (native protein, %) is observed. At 28 °C, after a month, the inulin-protected protein still had a 60% of native protein, while in the case of sucrose-protected protein, the native protein was preserved only 50%. All the samples had a doughy appearance after storage.

By the predictive study of activation energy, and using the data of native protein decrease for inulin and for sucrose at 10% (w/v) and for plasma without agent protective, the reaction exhibited first-order kinetics assessed at storage temperature for 30 days. The linear regressions were calculated for the protein concentrate samples at 28, 40 and 60 °C.

Some authors indicate that the reaction of the protein value loss in dehydrated foods follows first-order kinetics, in agreement with the results obtained in this work (Kaanane & Labuza, 1985; Labuza & Schmidl, 1985; Robertson, 1993). From the experimental data of Fig. 5, the values of k for each temperature were obtained (Figs. 6–8).

The calculation of protein concentrate activation energy (AE) might be carried out by considering natural logarithm of Eq. (3). From the slope of the lines the values were 7.12 cal/mol, 7.38 cal/mol and 7.35 cal/mol, from sucrose, inulin and plasma without agent protective, respectively, (Fig. 9).

The shelf life is defined as the storage time until 30% of undenatured protein remained. The products shelf life at different temperatures was calculated by Eqs. (2) and (3), considering 70% (w/v) and 30% (w/v) as initial and final quality factor respectively, and using the calculated AE value. For plasma concentrate without protectant agent the initial quality factor was 60% (w/v). The results are shown in Table 3. It can be seen that, as the storage temperature was increased, the product had a shelf life shorter than expected. It was found, at stored temperature of 2 °C, that a sucrose-protected protein concentrate had a shelf life of approximately 9 months, inulin-protected proteins concentrate had a shelf life of approximately 14.5 months, higher than that of the plasma without sugar. This longer shelf life period would be due to the fact that oligosaccharides have a lower tendency to crystallization than disaccharides and monosaccharides suggesting that oligosaccharides may be better stabilizers since they combine the advantageous characteristics of polysaccharides and saccharides for optimal protein stability (Hinrichs et al., 2005). Furthermore, studies of hygroscopicity of sugar glasses have demonstrated that the relative humidity produces T_g sharp drops, reflecting a plasticizing effect of water (Hinrichs et al., 2001). These authors proved that after exposure to air of 52% of humidity, no crystallization was observed for inulin, even after 3 weeks of storage. The excellent protective properties of inulin glass have been ascribed to the high T_g, poor hygroscopicity, low content of reducing groups and low crystallization rate.

3.5. Microstructure study

The micrographs of freeze-dried protein concentrate with and without inulin addition were analyzed by scanning electron

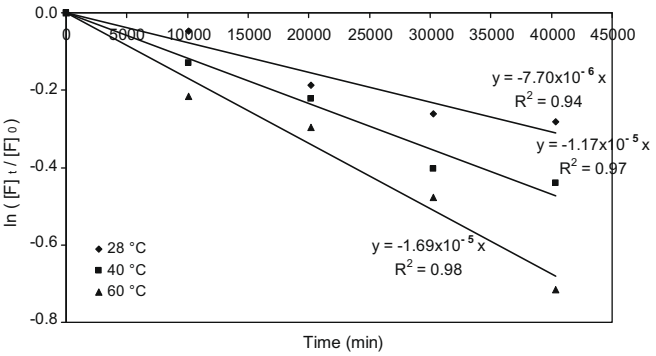

Fig. 6. Determination of the k value considering an order kinetics of $n = 1$, for proteins protected with sucrose at 10% (w/v). The error bars represent standard deviations.

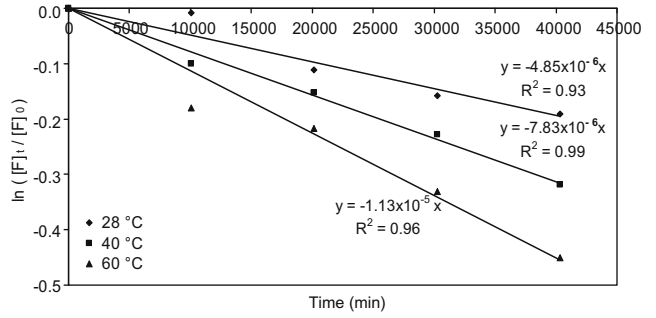

Fig. 7. Determination of the k value considering an order kinetics of $n = 1$, for proteins protected with inulin at 10% (w/v).

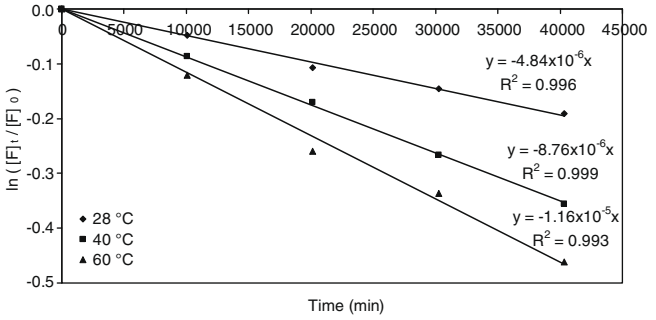

Fig. 8. Determination of the k value considering an order kinetics of $n = 1$, for proteins without protectant agent.

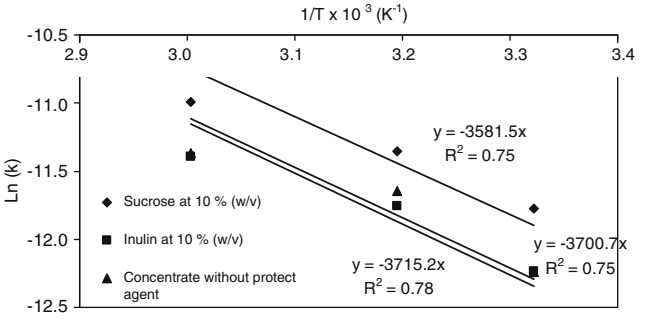

Fig. 9. Determination of activity energy for available native protein loss in the concentrates: protected with sucrose and inulin at 10% (w/v) and without protectant agent.

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Table 3
Prediction of the product shelf life without protective agent and with inulin and sucrose as a function of the decrease of the available native protein percentage.

Storage temperatures (°C)	Sucrose at 10% (w/v)		Inulin at 10% (w/v)		Plasma without protector	
	$k \times 10^6$ (min^{-1})	Half life (months)	$k \times 10^6$ (min^{-1})	Half life (months)	$k \times 10^6$ (min^{-1})	Half life (months)
2	2.21	9	1.36	14.5	1.43	11
15	3.97	5	2.50	8	2.63	6
20	4.91	4	3.11	6	3.27	5
30	7.35	2.5	4.73	4	4.96	3
50	15.30	1	10.10	2	10.6	1.5

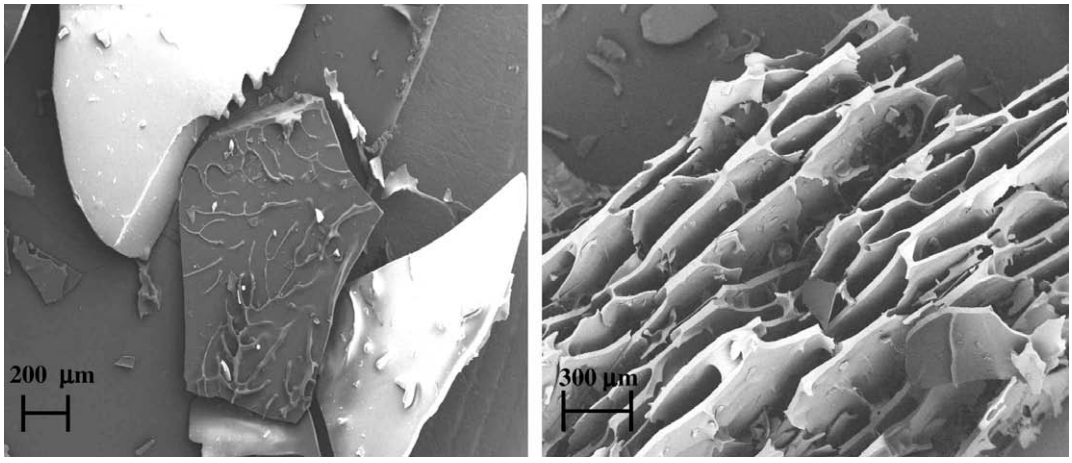

Fig. 10. Scanning electron micrographs of the freeze-dried product without protector solution: (a) superficial section microphotography, magnification 50×; (b) transversal section microphotography, magnification 50×.

microscopy (SEM). Fig. 10 shows the freeze-dried protein concentrate without the addition of a protectant agent. It can be observed a planar branched structure, without spatial folding, adopting a random structure. These presentation is characteristic of proteins which has undergoes a change or loss of conformation (Cheftel et al., 1989). On the contrary, Fig. 11 presents the freeze-dried protein concentrate with the lyoprotectant solution (inulin at 10% (w/v)). There, is not linear structures, but upon the surface appear globular structures with numerous aggregates with modular shape, similar to that previously founded by Rosell et al. (2009). The granular particles might be encapsulating proteins structure and this way preserving the native protein from denaturalization.

4. Conclusions

The protein concentrate obtained by UF–DD allows reducing the saline content and the denatured protein contain for the following treatments. The use of inulin exerted a protectant effect on the pro-

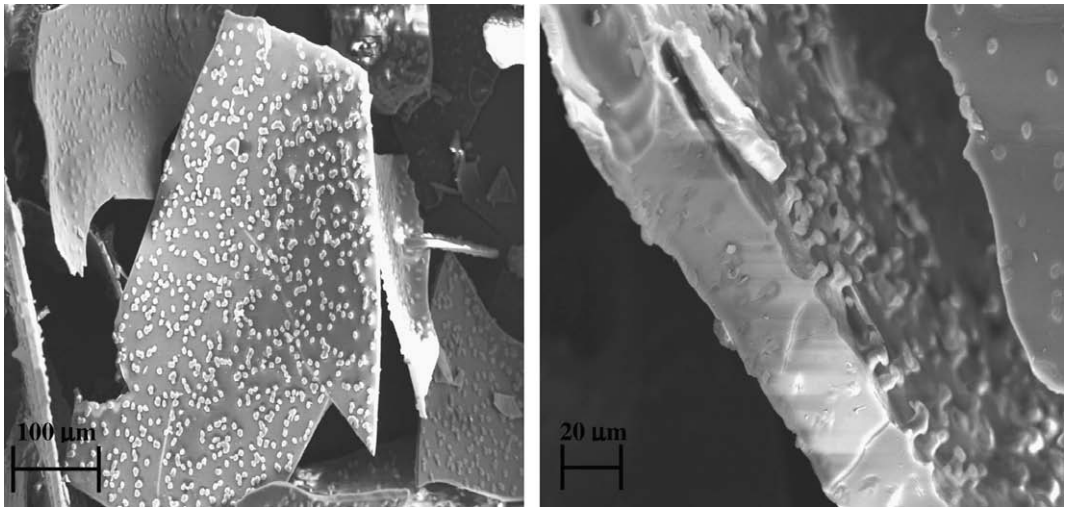

Fig. 11. Scanning electron micrographs of the freeze-dried product with protector solution of inulin at 10% (w/v): (a) superficial section microphotography, magnification 200×; (b) transversal section microphotography, magnification 700×.

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teins over the freeze-drying, still freezing at −20 °C a product of similar characteristics can be obtained, with a significant reduction of energy consumption. By this behavior, the denatured proteins percentage was reduced preserving their conformation, and consequently, their functional properties. Indeed, the concentrates with inulin has functional properties as solubility, emulsion and foam stability, improved with respect to the plasma without protectant agent.

The results showed that this oligosaccharide protect plasma concentrate during storage and had better stabilization properties than disaccharides such as sucrose to prevent the unfolding of bovine plasma proteins. A maximum storage time of 14 months at 2 °C was determined.

The final product combines the plasma proteins and inulin properties (Abrams et al., 2005; Hempel, Jacob, & Rohm, 2007; Nazzaro, Fratianni, Coppola, Sada, & Pierangelo, 2009), obtaining a functional ingredient to be used in food formulations with technological as well as health benefits. Furthermore, the formulation of protein concentrates as dry powder is an advantage with respect to storage and transportation and also, simpler dose modalities can be developed.

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