A new procedure to prepare transparent, colourless and low-water-soluble edible films using blood plasma from slaughterhouses

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Abstract

Some attempts to produce films using blood plasma proteins can be found in the literature; however, due to their high solubility in water, it is usually necessary to use crosslinkers, which may entail some disadvantages. In this work, a procedure to prepare water-insoluble edible films from bovine and porcine blood plasma without using crosslinkers is described for the first time, with the objective of producing sustainable packaging materials from livestock blood. For this purpose, the blood plasma fraction was acidified and treated with ethanol to precipitate the proteins, which were solubilised in water and mixed with glycerol in order to produce a film-forming solution. The generated films were investigated to determine light absorbance, transparency, microstructure, mechanical properties and solubility at several pHs and compared with a control film prepared with untreated plasma. The new films presented in this work were completely transparent and colourless on visual inspection, in contrast to the yellowish-orange colour of the control films. Furthermore, the microstructure of these new films was more homogeneous, and therefore they showed better mechanical properties than the control film. Finally, these films were found to be highly insoluble in buffer solutions of close to neutral pH, whereas the control film was almost completely solubilised in the same buffers.

1. Introduction

The most common proteins that have been typically used in film and coating formulations are collagen, gelatine, corn zein, casein, whey protein, wheat gluten and soy protein (Hassan, Chatha, Hussain, Zia, & Akhtar, 2018). Protein-based films exhibit poor resistance to water, i.e.

are prone to dissolve if the humidity of the medium is high enough; however, they are superior to polysaccharides in their capacity to form films with good mechanical and barrier properties (Mellinas et al., 2016).

The revalorisation of blood, or its fractions as rich protein sources, is strongly encouraged, since this co-product can be considered as one of the most problematic in the food industry due to the large amount that is generated and its high polluting power. In this sense, the use of blood plasma as a raw material for films potentially offers a way to minimise the environmental impact of blood generation while increasing the added value of blood proteins.

Elaboration and characterization of plasma protein-based films have been reported by several authors; Nuthong, Benjakul, and Prodpran (2009b) prepared films using porcine plasma previously dialyzed and lyophilized with glycerol as plasticizer. However, the films obtained showed a water solubility higher than 96%, which is highly undesirable for applications such as food coating or packaging. The same authors made several attempts to decrease the solubility of these films by adding crosslinkers, such as caffeic acid and glyoxal; however, glyoxal is a highly toxic compound and caffeic acid could exert a negative effect on the appearance of the films produced (Nuthong, Benjakul, & Prodpran, 2009a).

Taking all this into consideration and with the intention of overcoming such problems, in this study a new procedure, in which crosslinkers or other non-food-grade chemicals are not involved, is presented for the first time. This procedure is capable of preparing totally transparent and highly water-insoluble films from both bovine and porcine plasma proteins obtained from blood generated in a local slaughterhouse. In a preliminary analysis of the physical and functional properties of these films, they were tested and compared with those of a control film prepared by the traditional method.

2. Material and Methods

2.1. Blood Plasma Collection

Porcine and bovine blood was collected immediately after slaughtering from a local slaughterhouse (Asturias, Spain) and poured into 3 L plastic containers. Sodium citrate, previously added, at 2% (w/v) was used as an anticoagulant.

Plasma was separated from the cell fraction by centrifugation for 10 minutes at 10000 g and 10 °C. The plasma, which is the supernatant resulting from centrifugation, was decanted and stored at -20 °C.

2.2. Film Preparation

The procedure for the preparation of the new films from bovine and porcine plasma was the same in both cases. First, plasma was dialyzed, employing 14 kDa cellulose membranes (Dialysis tubing cellulose membrane, Sigma- Aldrich, United States) and then lyophilized and stored at a temperature of -20 °C until used.

Afterwards, 1.5 g of lyophilized plasma was dissolved in 50 mL of distilled water and the pH adjusted to 2.5, employing a solution of HCl 3.0 M. This acidified plasma solution was added dropwise to 400 mL of 96% ethanol (VWR, United States) and the pH adjusted to 1.5, once more employing HCl 3.0 M. The resulting mixture was centrifuged at 10000 g, at 10 °C for 30 minutes.

After centrifugation, the supernatant was discarded, and the pellet recovered. This pellet was dissolved in distilled water to concentration of 0.3 g/mL (w/v) by stirring at 600 rpm. Finally, 65 g glycerol/100 g of protein was added as plasticizer and 8.85 mg of protein/cm^2 of this film-forming solution was poured into Petri dishes. The films were dried in an oven at 40 °C overnight.

The control film was prepared according to Nuthong et al. (2009a) with slight modifications. Firstly, 30 g of lyophilized porcine plasma was dissolved in 100 mL of distilled water. Afterwards, the same glycerol ratio and drying procedure, previously reported, was used to prepare the control films.

The protein content in both the lyophilized untreated plasma and the pellet after the ethanol treatment, was determined by the Dumas combustion method using a CNHS/O Elementar Vario EL analyzer (Elementar, Germany).

2.3. Film Characterization

Prior to testing, films were equilibrated for at least 1 day in a desiccator at room temperature and with a controlled relative humidity of 54±2%. To maintain this relative humidity, a saturated solution of $Mg(NO_3)_2$ was placed at the bottom of the desiccator.

2.3.1. Light Transmission and Transparency

The light barrier properties of the films to visible and ultraviolet light were tested at different wavelengths following the method proposed by Saricaoglu, Tural, Gul, and Turhan (2018). The absorbance of the films was measured with an Analytik Jena Spekol® 1500 (LabWrench, Canada) from 200 nm to 600 nm. An empty quartz cuvette was used as a blank. Thickness of film samples was determined using a micrometer.

The transparency of the films is calculated according to the following equation:

$$Transparency = A_{600}/x \quad (1)$$

where A_{600} is the absorbance of the films at 600 nm and *X* is the thickness of the film in mm. The higher the *transparency* value obtained with this equation, the lower was the transparency of the film, and vice versa, the lower the *transparency* value calculated with this equation, the higher the transparency of the film.

2.3.2. Scanning electron microscope (SEM)

The microstructures of the film cross sections were analysed using a scanning electron microscope (SEM) (JSM-6610LV, JEOL, USA) according to the method described by Galus and Kadzińska (2016), with some modifications.

Firstly, the film samples were lyophilized and then cut into squares of approximately 1 cm^2 employing a surgical blade. These pieces were fixed to metal supports and gold coated in order to observe the cross-sectional microstructure of the films. The magnification used was x350 and the voltage was set at 20 kV.

2.3.3. Mechanical Properties

Mechanical properties of films were analysed employing a TA.XT. plus Texture Analyser (Stable Microsystems, United Kingdom), using a 50 N load cell and a 5 mm diameter probe (P/5S). To carry out the test, the films were cut into strips of 15x20 mm and placed between two plates which form part of the analysis device. These plates, firmly attached to the analyser, have an orifice of 10 mm that allows the probe to enter in contact with the film at a velocity 1 mm/s, stretching the film until it breaks.

The mechanical properties measured with this test were the puncture strength (*PS*) and the puncture deformation (*PD*). These properties were calculated according to the following equations:

$$PS = Fm/Th \quad (2)$$

$$PD = \left(\sqrt{D^2 + R^2} - R\right)/R \quad (3)$$

where *Fm* (N) is the maximum force applied before the film rupture, and *Th* (mm) is the film thickness ; *D* (mm) is the distance reached by the probe before the film is broken; and *R* (mm) is the radius of the orifice in the plates.

2.3.4. Film Solubility

Solubility was determined following the method proposed by Gontard, Duchez, Cuq, and Guilbert (1994) with slight modifications. Circular pieces of 1.9 cm were excised from the film and immersed in 20 mL of three different buffer solutions: Trizma® hydrochloride solution 0.1 M at pH 7.0 (Sigma-Aldrich, Estados Unidos), a carbonate-bicarbonate 0.1 M buffer solution at pH 9.0, and an acetic-acetate 0.1 M buffer solution at pH 5.0.

After 24 h of immersion, the circular pieces were recovered by employing a vacuum filtration system and Whatman Nº1 paper. The filter paper was weighed before proceeding with the filtration step. The filter paper and the film pieces were dried in an oven at 97 °C for 8 h.

On the other hand, intact pieces of film not previously immersed in the buffer solutions were directly dried at 97 °C for 8 h to determine the dry matter contained in the films. The amount of dry matter in the films recovered after the immersion in the buffers was compared with the same value of dry matter for the intact films to calculate the amount of film solubilized during the experiment.

2.4. Statistical Analysis

For data processing, an ANOVA test was used for variance analysis, and least significant differences (LSD) were calculated by Fisher's test to determine significant differences between the tested samples. The analyses were performed using Statgraphics® V.15.2.06 statistical software.

3. Results

3.1. Preparation of films, visual appearance, light transmission and transparency

Figure 1 illustrates the process of fabrication of the new blood plasma-based films. It was expected that the combination of both low pH and excess of ethanol would produce conformational changes in the plasma protein, and therefore lead to its aggregation and precipitation. In fact, after the centrifugation step, the supernatant had a clear, yellowish visual appearance, while the sediment showed a whitish aspect, which suggests that the plasma pigments and lipids were solubilised in the ethanol solution. The ethanol in the supernatant can be recovered with a rotary evaporator and reused. Electrophoresis analysis confirmed that all the main proteins of blood plasma were present in the sediment in the same proportion as they are found in the untreated blood plasma (data not shown). The dispersion of the recovered proteins in water resulted in a slightly viscous solution, which was then mixed with glycerol and dried to prepare the films.

The visual appearance of the films is also shown in Figure 1. After drying, the films were peeled easily from the Petri dishes, showed a homogeneous appearance and were sufficiently flexible to wrap a piece of food without breaking. Films prepared according to the novel method described in this paper (Figure 1B and 1C) were completely transparent and with no colour, whereas the control film had a yellowish-orange appearance, most likely due to the presence of bilirubin, carotenoids and haemoglobin, since all these compounds give the blood plasma its characteristic colour. The light transmission and transparency of the films tested are shown in Table 1. As was expected, the three films tested exhibited a high absorbance at 200 and 280 nm, mainly due to the absorption of light by carbonyl groups within the peptide bonds, the presence of aromatic amino acids that form part of the primary structure of the proteins, and disulphide bonds (Banga, 2015). This property is common to any protein-based film, and it is very desirable for packaging applications, since it can act as a barrier to UV radiation, hindering the UV-mediated oxidative degradation of the lipids that can occur in many food items (Wiegand et al., 2018). When analysing the absorbance in the visible range, this was significantly higher for the control films in the entire range. This effect may be explained because the pigments and compounds that are found in the untreated plasma, and which subsequently constitute part of the control film, exhibit absorbance and light scattering at those wavelengths. Such compounds were removed by the ethanol in the new film making process. Finally, although both treated and untreated films were found to be highly transparent, the least transparent was the control, with a transparency value of 0.50, in contrast with transparency values of 0.10 and 0.21 for the

porcine and bovine films, which clearly shows that this characteristic of the films was improved by the new process.

3.2. Microstructure and mechanical properties

The microphotographs of the films' transversal sections are shown in Figure 2. In this case, the new films (Figure 2B and 2C) showed a more homogeneous and compact microstructure than the control films (Figure 2A). When the film-forming solution is drying, the protein chains are approaching each other and forming, mainly, inter- and intramolecular non-covalent bonds, namely hydrophobic interactions, hydrogen bonds and ionic interactions. In any case, the treatment applied to the plasma to prepare the new films produces a partial denaturalization of the plasma proteins, leading to the exposure of their hydrophobic cores. Hence, when the solvent of the film-forming solution is evaporating, these proteins can interact through a higher number of hydrophobic bonds and thereby increase the degree of packing of the film matrix. Furthermore, it must be remembered that most of the blood-plasma compounds remain in the control film, where some of these compounds may hamper the approximation of the protein chains, decreasing the number of non-covalent bonds.

3.3. Mechanical properties

The mechanical properties (Table 1) seem to be closely related to the microstructure of the films. In this sense, the more homogeneous the film microstructure is, the better are the mechanical properties. In this case, the values of the *PS* and *PD* parameters were significantly higher for the new films when compared to the control. Among the new films, the porcine based ones showed the best *PS* values, while bovine based ones showed the highest *PD* readings, although the differences detected could be considered minor considering the mean values obtained for these parameters. These small differences may be produced due to slight changes in the amino acid sequence between bovine and porcine plasma proteins, which mainly involve some amino acid substitutions in the main protein fraction (immunoglobulin). Such small differences may lead to different final properties of the films, as shown in this work.

3.3. Water solubility (WS)

The WS values of the tested films are shown in Table 1. WS is an important assessment parameter for novel films prepared using natural biopolymers. It must be borne in mind that many food products have relatively high moisture levels, and this water content might damage the integrity of the film, which would diminish film performance, and therefore, its range of

applications. Because of the biological function and biochemical properties of plasma proteins, they have high water solubility at physiological pH, so the films prepared using untreated plasma are expected to be solubilised to a large extent when they are immersed in the buffer solution at pH 7.0. In this case, the control sample was almost completely solubilised after 24 h of exposure at this pH, which is in agreement with the findings of other authors (Nuthong et al., 2009b). In addition, the control film was also almost completely solubilised at pHs 5.0 and 9.0.

On the other hand, the new films prepared here showed a significantly lower water solubility at the same testing conditions. In fact, the lowest WS values were obtained at pH 7.0, the solubility of the new porcine plasma film being 8.8 ± 3.1% of its total dry matter, and that of the new bovine plasma film 11.6 ± 5.5% of its total dry matter. However, the amount of film solubilised increased noticeably at pH 5.0, reaching WS values of 21.2 ± 2.9% and 26.1 ± 3.4% for the new porcine and bovine plasma films respectively; and to a lesser extent at pH 9.0, showing in this case WS values of 15.0 ± 0.3 and 17.0 ± 2.7 for the new porcine and bovine plasma films respectively. These findings suggest that the acidification of the plasma protein and its precipitation in ethanol produced a variation in the solubility profile of these proteins, causing these films to become mostly insoluble at pH levels close to neutrality. In regard to this, it has to be said that most food products have a pH that is slightly acid or close to neutral, so it is expected that these new films will be suitable for wrapping a wide range of foodstuffs.

3. Conclusions

A successful new procedure to produce films using bovine and porcine blood plasma collected from slaughterhouses was described. These new films performed significantly better in key parameters such as colour, transparency and mechanical strength when compared to other methods described previously. They also showed low solubility in buffer solutions at different pH values relevant for most food products. Furthermore, it was proven that if the plasma is treated in this way, the films produced show a more homogeneous and compact matrix microstructure. Finally, in spite of the high consumption of ethanol, there is no doubt that it could be easily recovered in order to minimise reagent usage, thus making the process more sustainable.

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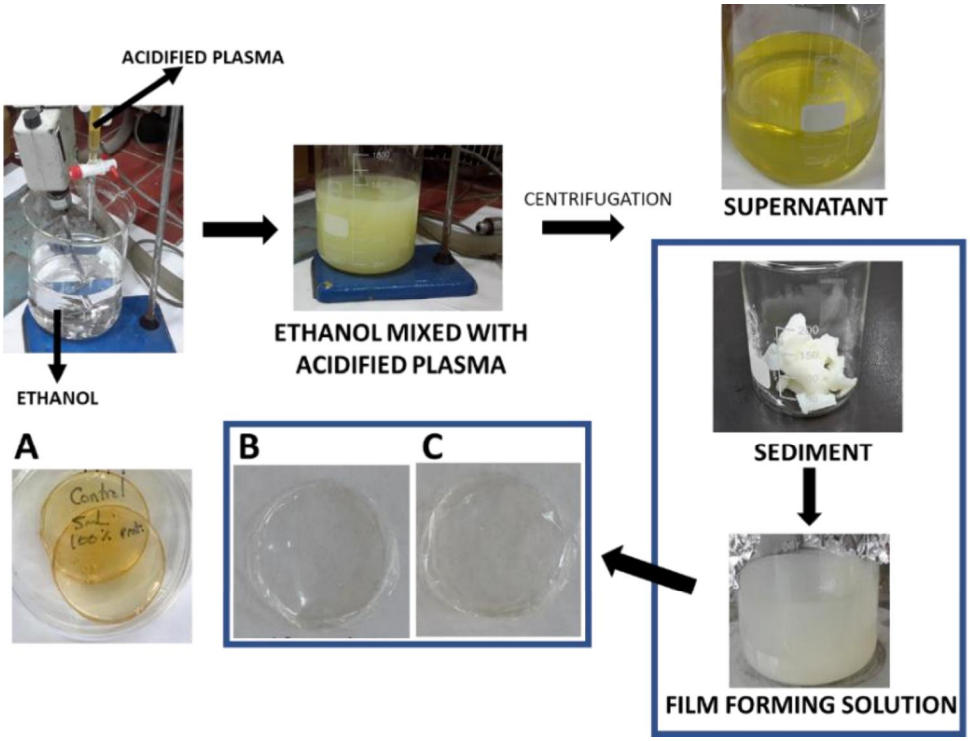

Figure 1. Fabrication process of the new plasma films and their visual appearance. visual appearance of the control film (A), and the new films prepared from bovine (B) and porcine (C) plasma.

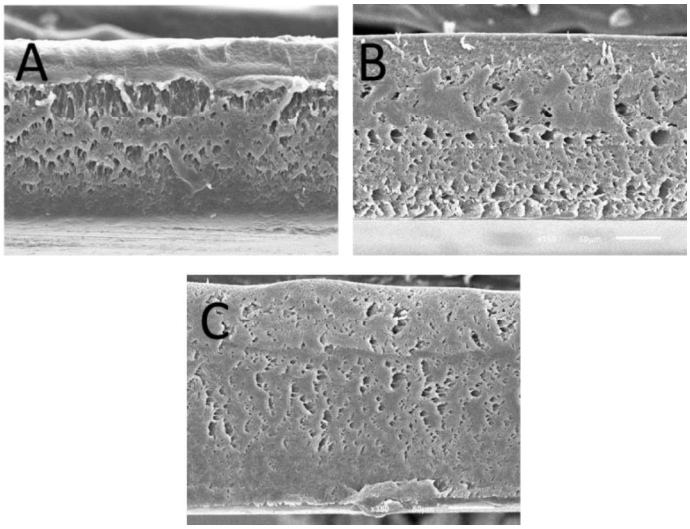

Figure 2. Microphotographs of the transverse section of the control film (A), the new plasma film from bovine blood (B) and the new plasma film from porcine blood(C).

312 Table 1. Thickness, puncture strength *(PS),* puncture deformation *(PD),* water solubility (WS), absorbance and *transparency* of the tested films.

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	Thickness (mm)	*PS* (N/mm)	*PD* (%)	WS (%)			Absorbance (Wavelength, nm)							*Transparency*
				5.0	7.0	9.0	200	280	300	350	400	500	600	
Control	0.179 ± 0.004[a]	23.4 ± 5.1[a]	12.3 ± 2.5[a]	96.0 ± 1.1[a]	95.2 ± 3.5[a]	97.1 ± 2.1[a]	3.00	3.00	2.10	0.36	0.30	0.23	0.09	0.50
New porcine film	0.168 ± 0.010[ab]	47.0 ± 1.9[b]	22.4 ± 3.8[b]	21.2 ± 2.9[b]	8.8 ± 3.1[b]	15.0 ± 0.3[b]	2.53	2.70	1.62	0.06	0.06	0.02	0.01	0.10
New bovine film	0.160 ± 0.012[b]	42. 2 ± 1.6[c]	30.3 ± 1.2[c]	26.1 ± 3.4[b]	11.6 ± 5.5[b]	17.0 ± 2.7[b]	2.20	2.45	1.58	0.08	0.05	0.04	0.03	0.21

314 Different letters in the same columns indicate significant differences ($P < 0.05$).

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Sara Álvarez: Investigation, Writing-Original Draft.

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