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Soy protein – Poly (lactic acid) bilayer films as biodegradable material for active food packaging☆

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ABSTRACT

The preparation and characterization of biodegradable bilayer films from isolated soy protein (SPI) and poly (lactic acid) (PLA) were carried out in this work. The films showed high transparency and strong adhesion between layers without adding an extra component, or without chemically modifying the film surfaces. The application of the PLA layer largely increased the mechanical properties of the films with respect to those of pure SPI films. Furthermore, the hydrophobic characteristics of the PLA layer improved film performance under conditions in which water was involved, markedly decreasing the amount of total soluble matter, the swelling index and the water vapor permeability. The biodegradation under soil burial conditions was evaluated measuring weight loss as a function of time, showing a two-step degradation and a faster degradation rate for the protein component compared to those of PLA layer. The films prepared were evaluated as active packaging by incorporation of an antifungal and an antibacterial agent (natamycin and thymol, respectively) to the SPI layer, showing a marked growth inhibition of mold, yeast and two strains of bacteria by in-vitro microbiological assays.

1. Introduction

Most plastic containers currently used for food packaging are made up of petroleum-based polymeric materials. Their use is widespread in this and many other applications, due to their numerous advantages, including large scale availability, relatively low production cost, lightweight, versatile and good mechanical and barrier properties (Tharanathan, 2003).

However, these materials have certain disadvantages since, in addition to being synthesized from a non-renewable source, they are not biodegradable, proving a major source of generation and accumulation of residues (Bucci, Tavares, & Sell, 2005).

At present, the bio-polymer concept is emerging. Biodegradable materials are associated with the use of renewable raw materials such as proteins and polysaccharides extracted from agricultural, marine, animal or microbial sources. These materials can be degraded by the environment (exposed to soil optimum moisture, microorganisms and oxygen) into simple substances (water and carbon dioxide) and biomass.

However, materials from natural polymers are associated with poor mechanical and "barrier" properties and low thermal stability. For this reason, researchers need to develop synthetic modification strategies to improve their poor properties. Some strategies are based on blends with synthetic polymers (Tian, Wang, Zhang, Quan, & Zhang, 2010), lipids (Monedero, Fabra, Talens, & Chiralt, 2009, 2010) (increasing the hydrophobicity of the film) or other biodegradable polymers with different properties (Abugoch, Tapia, Villamán, Yazdani-Pedram, & Díaz-Dosque, 2011). The use of plasticizers and chemical modifications like cross-linking (Bigi, Cojazzi, Panzavolta, & Rubini, 2001; González, Strumia, & Alvarez Igarzabal, 2011; Vaz, 2005) and grafting with vinyl monomers (Hai-Ping, Bao-Wang, Si-Min, Li-Ming, & Hu-Zeng, 2010; Yang, Xuegang, Xiaoyan, & Chi, 2011) are other widely used strategies.

The isolated soy protein (SPI) as raw material has shown advantages over other sources due to its exceptional film-forming properties, low cost (for its extensive production in our country and for being isolated from oil industry waste) and good barrier properties to oxygen, aromas and lipids under low to intermediate moisture conditions (Gennadios, Weller, & Testin, 1993). This type of proteins also produces softer, more transparent and flexible films compared to those derived from other sources (Guilbert, Gontard, & Cuq, 1995).

☆ In memory of Ignacio G. Studer.

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However, SPI contains 58% polar amino acids that cause hydrophilicity (Choi, Kim, Hanna, Weller, & Kerr, 2003) leading to brittleness in its wet state and poor moisture barrier and mechanical properties.

These effects can be minimized by physical or chemical treatment. On the other hand, poly (lactic acid) (PLA) is a biodegradable material whose use in food packaging is being currently developed. Some studies indicate that this material is equally suitable for such applications compared to the widely used low density polyethylene (Koide & Shi, 2007).

Recently, PLA composites comprising other polymers or inorganic materials have been extensively investigated to reduce material costs. For this purpose, natural polymers such as fibrous cellulose, starch, soy protein and other agricultural residues are often used as the inexpensive renewable polymers that form composites with PLA (Bo, Long, Hongzhi, & Jinwen, 2010).

In this study, two-component bilayer films (PLA and SPI) were developed, between which physical type interactions were found (hydrogen bond type interactions between PLA carbonyls and the hydrogens of the peptide bonds of protein).

One of the main research areas in food packaging has focused on developing new packaging techniques capable of improving food preservation properties based on their interaction with packaging. Such techniques are known as “active packaging systems”. An active packaging can be defined as a type of material that changes its packaging conditions to extend shelf life, interacting directly with the food, enhancing security and maintaining quality.

In particular, the antimicrobial packaging is one of the most innovative and promising active packaging types developed over the last decade, which includes systems capable of inhibiting microorganism action and avoiding loss of food quality (Seydim & Sarikus, 2006). Previous literature has shown that protein coatings on films in composite structure would require relatively lower amounts of added antimicrobial agents to reach the desired effect as compared to the synthetic polymer or to other biopolymer films (Chaa, Cookseyb, Chinnana, & Park, 2003). In the present research work, natamycin and thymol were incorporated into SPI–PLA films.

2. Materials and methods

2.1. Materials

The following chemicals were used: soy protein isolate SPI SUPRO E with 90% protein on fat-free, dry-weight basis (donated by The Solae Company, Argentina), poly (lactic acid) PLA (Cargill), glycerol (Taurus, analytical grade, ACS), sodium hydroxide and calcium chloride (Cicarelli), chloroform (Anedra), natamycin (Proquiga S.A.), thymol (Anedra). Strains of bacteria (*Staphylococcus aureus and Escherichia coli*), mold (*Aspergillus* sp.), and yeast (*Saccharomyces cerevisiae*) are belonging to the culture collection of the Centre for Applied Chemistry (CEQUIMAP). In microbiological tests, Mold and Yeast and Brain-heart agar were used.

2.2. Bilayer film preparation

Films were prepared using different proportions (%) of SPI/PLA: SPI 100, 60/40 and 50/50. SPI films were prepared by the “casting” method, adding 0.6 g of SPI powder to 80 mL of deionized water under constant stirring. The pH was adjusted at 9 with NaOH and glycerol (50% by mass with respect to the mass of SPI) was added as plasticizer. The dispersion was stirred for 1 h at 90 °C (at this temperature the protein denaturation is promoted). The dispersion was placed in a polypropylene recipient and dried in an oven at 55 °C for 12 h. The PLA layer was then prepared above the dried SPI layer. For this, 0.4 and 0.6 g of PLA (for 60/40 and 50/50 films, respectively) were dissolved in 50 mL of chloroform and stirred for 1 h. The solution was poured into the recipient containing SPI layer and finally dried for 4 h at room temperature.

2.3. FT-IR-ATR analysis

In order to confirm the chemical nature of the film components (SPI and PLA), the two faces of 60/40 and 50/50 films were analyzed by Fourier Transform Infrared Spectroscopy in Attenuated Total Reflectance mode (FTIR – ATR) using a ZnSe crystal with an incidence angle of 45 grades. Conventional transmission IR analysis was also carried out.

Different clean areas of three samples were analyzed to confirm the homogeneity of each film. All spectra represent the average of 42 scans recorded at 4 cm^{-1} resolution in a 4000 to 400 cm^{-1} range, using air as background.

2.4. Film thickness

Thickness was determined as the average of 10 measurements for each sample with a hand-held micrometer (Schwyz model ESP1-0001PLA, Schwyz, Swiss). The average film thickness was used for assessing opacity, water vapor permeability and mechanical properties.

2.5. Opacity

Each film was cut in a 2.5 × 1 cm rectangle; opacity was determined as the area under the absorbance curve in the visible spectrum ($\lambda = 400–800$ nm) of each sample. The value of the areas was normalized, divided by the thickness of each film.

2.6. Contact angle analysis

The contact angle measurements performed on the samples were carried out by the Sessile Drop method using a homemade contact angle goniometer (Romero, 2012). The contact angles were measured carefully from the left and right sides of the drop and subsequently averaged. The liquid used was distilled water and the experiments were performed at room temperature.

2.7. Swelling index (S)

The film water sorption capacity was determined by immersing known masses of each film in 30 mL of distilled water at room temperature for 30 min. The weight variation between the swollen and the dried state was measured. The films were superficially dried with absorbent paper to remove water excess before each weight operation. This assay was performed in triplicate. Eq. (1) shows how to calculate *S*, where m_h and m_i are the swollen and the initial weight, respectively.

$$S = [(m_h - m_i)/m_i] \times 100 \quad (1)$$

2.8. Moisture content (MC) and total soluble matter (TSM)

Moisture content (MC) was determined according to a method described in the literature (Rhim, Gennadios, Weller, & Hanna, 1998). Film samples were weighted (W_0) into glass dishes, dried in an oven at 110 °C for 24 h and weighted again (W_i). Moisture content for each film was determined in quadruplicate by Eq. (2).

$$MC = [(W_0 - W_i)/W_0] \times 100 \quad (2)$$

Total soluble matter (TSM) was determined according to a method described in the literature (Rhim et al., 1998). Dry and

soluble matters were measured on different films from each cast film to avoid cross-linking by heating of the samples prior to incubation in water. Four weighted samples of each film were immersed in beakers containing 30 mL of distilled water. The beakers were stored in an environmental chamber at 25 °C for 24 h with occasional stirring. The insoluble matter was then separated and dried in an oven at 110 °C for 24 h (W_f) to determine the solubilized dry matter by Eq. (3). The measurements for each type of film were obtained in quadruplicate. Initial dry matter values needed for TSM calculations were obtained from MC measurements for a film with the same mass (W_i). The reason for using different film specimens to measure initial and soluble dry film matter is that the proteins are susceptible to heat-induced cross-linking and this effect would decrease the TSM of the films (Gennadios, Ghorpade, Weller, & Hanna, 1996).

$$TSM = [(W_i - W_f)/W_i] \times 100 \quad (3)$$

2.9. Water vapor permeability (WVP)

Water vapor permeability (WPV) was determined in duplicate for each film following the procedure of desiccant, described by a standard method (ASTM Standard E96M-10, 2010). The films were placed in a humidity chamber at 25 °C and 65% relative humidity (RH) for a period of 2 days to reach equilibrium. Subsequently, these films were fixed onto aluminum capsules (50 mm diameter, 17 mm depth) containing anhydrous $CaCl_2$ (dried at 180 °C for 24 h), sealed with silicone grease. The desiccant was separated from the atmosphere by the film. These capsules were weighed and placed in a humidity controlled chamber under the same conditions as those in which the films were previously conditioned. The weight variation of the entire system was recorded every one hour up to 9 measurements. These values were plotted as weight variation versus time, obtaining a linear characteristic graph. Water vapor transmission (WVT) was calculated using Eq. (4):

$$WVT = F/A \quad (4)$$

where F and A are the slope of the linear graph and surface area exposed, respectively. WVP is then calculated according to Eq. (5):

$$WVP = (WVT \times e)/[S \times (RH1 - RH2) \times 3600] \quad (5)$$

where e is the film thickness, S is the saturation pressure at 25 °C and (RH1−RH2) is the difference in relative humidity (RH) between the interior and exterior of the capsule.

2.10. Mechanical properties

In these analyses, each sample was cut into 25 × 100 mm pieces and stress–strain curves were performed. Tensile strength (TS), elongation at break (EB) and Young's modulus (E) were determined according to a standard method (ASTM Standard D882-02, 2002). An Instron Texturometer (model 3342, Norwood, MA, USA) equipped with a 500 N cell was used at a 0.5 mm/s speed. For each sample, five measurements were made and averaged.

2.11. Thermal properties

Prior to the assays, the film samples were dried in a vacuum chamber. Differential scanning calorimetry (DSC) analyses of the films were performed on a 2920 Modulated DSC (TA Instruments). For each film sample, about 2.5 mg was sealed in an aluminum pan and heated from 25 to 100 °C at a rate of 10 °C/min and then cooled to 25 °C before a second heating scan to 200 °C at a 10 °C/min scan rate. A nitrogen flow (60 mL/min) was maintained during the entire test. Glass transition temperature (Tg), melting temperature (Tm) and enthalpy of fusion (Hf) were determined from the second heating scans. The thermogravimetric analysis (TGA) was performed in a Hi-Res Modulated 2950 Thermogravimetric Analyzer (TA Instruments) using the same temperature program as DSC.

2.12. Biodegradation test

The biodegradation tests were carried out by a method described in the literature (Martucci & Ruseckaite, 2009). Equal masses of each film were buried in a characterized soil for 75 days in a closed environment. The most relevant physico-chemical properties of the soil were: organic matter: 10%; organic carbon: 5.80%; total nitrogen: 0.464%; C/N ratio: 12.5; nitrates: 128.0 ppm; sulfates: 23.3 ppm; phosphorus: 58.7 ppm; pH: 7.14 and electrical conductivity of saturation extract: 3.1 dS/m. The samples were cut into rectangular pieces, dried in an oven at 105 °C for 12 h and weighed (W_0). The films were then buried into an iron mesh (to allow access of moisture and microorganisms and to facilitate removal of the degraded samples) in plastic boxes ($100 \times 20 \times 15$ cm^3) at a depth of 8 cm from the soil surface in order to ensure aerobic degradation conditions. The assay was performed at (21 ± 2)°C and (39.5 ± 4)% RH by adding water periodically. Fluctuations in soil moisture were followed gravimetrically using the standard method of oven drying. Samples were taken from the soil at different times and cleaned by wiping gently with a brush. Subsequently, they were dried in an oven at 105 °C for 12 h and weighed (W_t) to assess average weight loss (%WL). All determinations were performed in triplicate. Eq. (6) was used to obtain %WL values:

$$\%WL = [(W_0 - W_t)/W_0] \times 100 \quad (6)$$

2.13. Incorporation of active agents into the films

Thymol and natamycin were incorporated in the SPI layers. The films were prepared as in procedure 2.2, but adjusted to pH = 9. The active agents were directly added to the SPI dispersions in water after heating once room temperature was reached. Different quantities of antibacterial and antifungal agents were used (2.5; 5; 10; 15; 20; 25 and 50% w/w of SPI for thymol and 0.33 and 0.52% w/w of SPI for natamycin).

Film drying was performed in an oven for 12 h at 45 °C and 72 h at room temperature for natamycin and thymol-containing films, respectively. PLA layer was prepared using the same procedure as detailed in Section 2.2.

2.14. Behavior on food

Four pieces of three selected foodstuffs like apple, tomato ($3 \times 3 \times 1$ cm^3) and soft cheese ($6 \times 4 \times 1$ cm^3) were packed with different types of wrapper. The coatings used were: SPI–PLA 60/40 film, SPI–PLA 60/40 with natamycin and a commercial polypropylene container. Packaged foods were stored in a refrigerator at 10 °C. Subsequently, each piece was observed and photographed at different times.

2.15. In-vitro microbiological assays

Inhibition assays of mold, yeast and bacteria (Gram-negative and Gram-positive) were performed. A strain of *Aspergillus* sp mold, *S. cerevisiae* yeast, *E. coli ATCC 25922 and S. aureus ATCC 25923* strain were used. The inhibition assays were carried out using the agar

diffusion test. The active films were cut into 16 mm diameter discs, arranged in Petri dishes containing Mold and Yeast agar to assess inhibition of fungal flora, and Brain-heart agar to assess inhibition of bacterial growth. SPI–PLA 60/40 films with natamycin, thymol and a release control (without active agent) were used. Two experimental designs were used. In the first one, the films with the active face down were placed on the corresponding culture medium, previously inoculated with the microorganism by raking. Each inoculum was previously prepared by suspension in a phosphate buffer dilution of the respective microorganism, taken from a fresh pure culture of up to 48 h of incubation after ringing. In a second experimental model, film discs were embedded in the agar. On a solidified layer of the corresponding culture medium, the films were placed with the active face upwards. A second layer of culture medium kept at a temperature slightly above the melting point, and previously inoculated with the microorganism suspension to be evaluated, was poured over them.

Both experimental designs were used in order to observe the results obtained.

2.16. Statistical analysis

Data for each test were statistically analyzed. The analysis of variance (ANOVA) was used to evaluate the significance in the difference between means. Turkey test was used for comparing mean values. Differences between means were considered significant when $P \leq 0.05$.

3. Results and discussion

Different films were obtained, prepared by the "casting" method (SPI 100 and SPI–PLA in 60/40 and 50/50 proportions). The synthesized films showed high stability between layers since they were not separated in any determination performed in the work. The bilayer films showed to be heat-sealable using a traditional thermo sealing instrument. This property represents an important technological advantage for the utilization of this material as packaging. The prepared films were physico-chemically characterized by using different characterization techniques, some of which were previously used in the research group (Aldana, González, Strumia, & Martinelli, 2012; González et al., 2011).

3.1. FT-IR-ATR analysis

The chemical characterization of both sides of the SPI–PLA 50/50 film was carried out by FT-IR-ATR. This technique allows obtaining the spectra of both surfaces of the film. Spectra with different characteristics were obtained for each layer, in which the specific bands of the pure compound can be observed.

The characteristic absorption peaks observed for the SPI layer were: at 3000–3500 cm^{-1} (O–H and N–H stretching which are able to form hydrogen bonding with the carbonyl group of the peptide linkage); 2928 cm^{-1} (CH_2 asymmetrical stretching); 1644 (C=O stretching, amide I); 1536 cm^{-1} (N–H bending, amide II); 1398 cm^{-1} (N–H bending, amide III); 1234 cm^{-1} (C–N stretching, amide III) and 1104 cm^{-1} (C–O stretching).

In the PLA layer spectra, the following peaks were observed: a weak absorption at 3500 cm^{-1} (O–H stretching of PLA end hydroxyl groups); 2994 cm^{-1} (C–H stretching of $-CH_3$); 1748 cm^{-1} (C=O stretching); 1454 cm^{-1} (C–H stretching of $-CH_3$; asymmetric deformation); 1382 cm^{-1} (C–H stretching of $-CH_3$; symmetric deformation); 954 cm^{-1} (O–H vibration of carboxylic acid) and both at 1186 and 1078 cm^{-1} (C–O stretching). The same characteristic bands were reported in the literature (Bo et al., 2010; González et al., 2011; Guerrero, Retegi, Gabilondo, & de la Caba, 2010; Guerrero, Stefani, Ruseckaite, & de la Caba, 2011; Jiménez Bonilla, Sibaja Ballestero, & Vega–Baudrit, 2012; Soliman, Tawfic, El-Saied, & Moharram, 2007).

Thereby, it was possible confirm the chemical nature of each component. It was also found that, during the preparation, both polymers did not mix each other since the spectra obtained from each layer corresponded to the pure component spectra. In addition, a conventional IR spectrum was performed across the film, in which the two film layers were represented, since the characteristic bands of both components can be seen.

Table 1
Opacity values of the different films.

Film	Opacity (UA/μm)
SPI 100	1.35 ± 0.20^{b}
SPI–PLA 60/40	0.82 ± 0.05^{a}
SPI–PLA 50/50	0.71 ± 0.12^{a}

Any two means in the same column followed by the same letter are not significantly ($P \geq 0.05$) different according to Turkey test.

3.2. Film opacity

The samples were physically characterized, determining the opacity of each film. Table 1 shows the results. It is well known that PLA forms transparent films (Martino, Jimenez, & Ruseckaite, 2009). The values obtained in the prepared films were particularly low, which represents the notable transparency of the films. Moreover, PLA-containing films were more transparent than SPI film. The low opacity of the bilayer films conveys a sense of great stability achieved between the two phases.

3.3. Contact angle analysis

The contact angle is a measure of the wettability of a surface by a liquid. It broadly defines the hydrophilic/hydrophobic character of the surface.

The results of contact angle determinations measured on both sides of the films were:

$$\text{SPI } 100 : (47 \pm 5)^{\circ}$$
$$\text{SPI–PLA } 60/40, \text{ SPI surface} : (47 \pm 2)^{\circ}$$
$$\text{SPI–PLA } 60/40, \text{ PLA surface} : (82 \pm 2)^{\circ}$$

Hence, it can be seen that the PLA layer is substantially more hydrophobic than SPI and the presence of the PLA layer in SPI–PLA films does not practically affect the value of the contact angle of the SPI surface as compared to the SPI 100 film.

The hydrophobicity of the PLA layer exhibits a different behavior against the action of water and gives improvements to the PLA-containing films compared to the SPI 100 film, as analyzed in the following tests.

Table 2
Swelling index (S), total soluble matter (TSM), water vapor permeability (WVP) and moisture content (MC) of the different films.

Film	S (%)	TSM (%)	WVP ($.10^{-11}$ g m Pa^{-1} s^{-1} m^{-2})	MC (%)
SPI	1005.4 ± 56.2^{b}	83.4 ± 10.4^{b}	14.9 ± 0.5^{a}	32.3 ± 2.3^{b}
SPI–PLA 60/40	209.3 ± 10.8^{a}	32.0 ± 15.2^{a}	3.4 ± 0.1^{b}	27.5 ± 2.5^{b}
SPI–PLA 50/50	185.4 ± 12.4^{a}	40.1 ± 5.0^{a}	2.3 ± 0.1^{c}	18.5 ± 3.4^{a}

Any two means in the same column followed by the same letter are not significantly ($P \geq 0.05$) different according to Turkey test.

Table 3
Elongation at break (EB), tensile strength (TS), Young's modulus and thickness of the SPI and SPI–PLA films in different proportions.

Film	EB (%)	TS (MPa)	E (MPa)	Thickness (μm)
SPI 100	24.63 ± 0.13^{b}	1.08 ± 0.34^{a}	22.80 ± 6.14^{a}	45 ± 8^{a}
SPI–PLA 60/40	1.09 ± 0.09^{a}	8.57 ± 1.61^{b}	1085 ± 134^{b}	51 ± 6^{a}
SPI–PLA 50/50	1.25 ± 0.02^{a}	13.69 ± 0.94^{c}	1579 ± 52^{c}	54 ± 5^{a}

Any two means in the same column followed by the same letter are not significantly ($P \geq 0.05$) different according to Turkey test.

3.4. Swelling index (S), moisture content (MC), total soluble material (TSM) and water vapor permeability (WVP) analysis

In the material characterization (Table 2), the effect of variation in the hydrophilicity of the films on certain relevant properties in which the influence of water is involved, was analyzed. As expected, we observed a marked decrease in the properties studied as *S*, MC, TSM and WVP, because the increase in the PLA proportion decreases the affinity of the material by water.

Performing the swelling index study, it was found that, when the films kept in contact with water, they showed a softening state in a first stage, followed by an increase in swelling. Moreover, in all cases, the swelling increased sharply in the first minutes and then began to decrease since part of the film was probably lost by solubilization during the assay. A similar behavior was found for cross-linked SPI films in previous works (González et al., 2011). The results showed a marked difference in swelling between SPI 100 and SPI–PLA films, in which a significant decrease in *S* with the increase in PLA fraction was also found. This behavior was also reported in the literature for SPI/PLA blends prepared by extrusion (Zhang, Jiang, & Zhu, 2006).

In addition, the amount of total soluble matter (TSM) for each film was studied. Solubility in water is a property which governs potential applications of these materials to food preservation. Films with low water solubility are necessary for the protection of foodstuffs with high or intermediate water activity (aw) (Sébastien, Stéphane, Copinet, & Coma, 2006).

In the present work, it was found that the amount of total soluble matter (TSM) for PLA-containing films was much lower than that of SPI 100 film. This decrease depicts a marked improvement in the material behavior when placed in contact with water.

Furthermore, it is known that PLA has good barrier properties to water vapor (Siparsky, Voorhees, Dorgan, & Schilling, 1997). This could be determined in the permeability analysis of the films, where the decreases in the values were obtained after the addition of the PLA layer. Thus, lower water vapor permeability values were obtained for the bilayer films compared to the SPI film. Literature describes a multilayer system which exhibits a similar effect produced by PLA layers in protein films (gelatin) (Martucci & Ruseckaite, 2010). In this work, the effect is more marked (greater decreases) since two PLA layers are covering the protein layer forming a three-layer film.

3.5. Mechanical properties

Tensile tests were conducted for the films prepared, and stress–strain curves were performed. From these graphs, the following mechanical properties were determined: tensile strength (TS), Young's modulus (*E*) and elongation at break (EB). From Table 3, can be seen that the presence of PLA and the increase in its proportion produced a decrease in EB and a marked increase in TS and E, which means that the material becomes less elongable and more resistant,

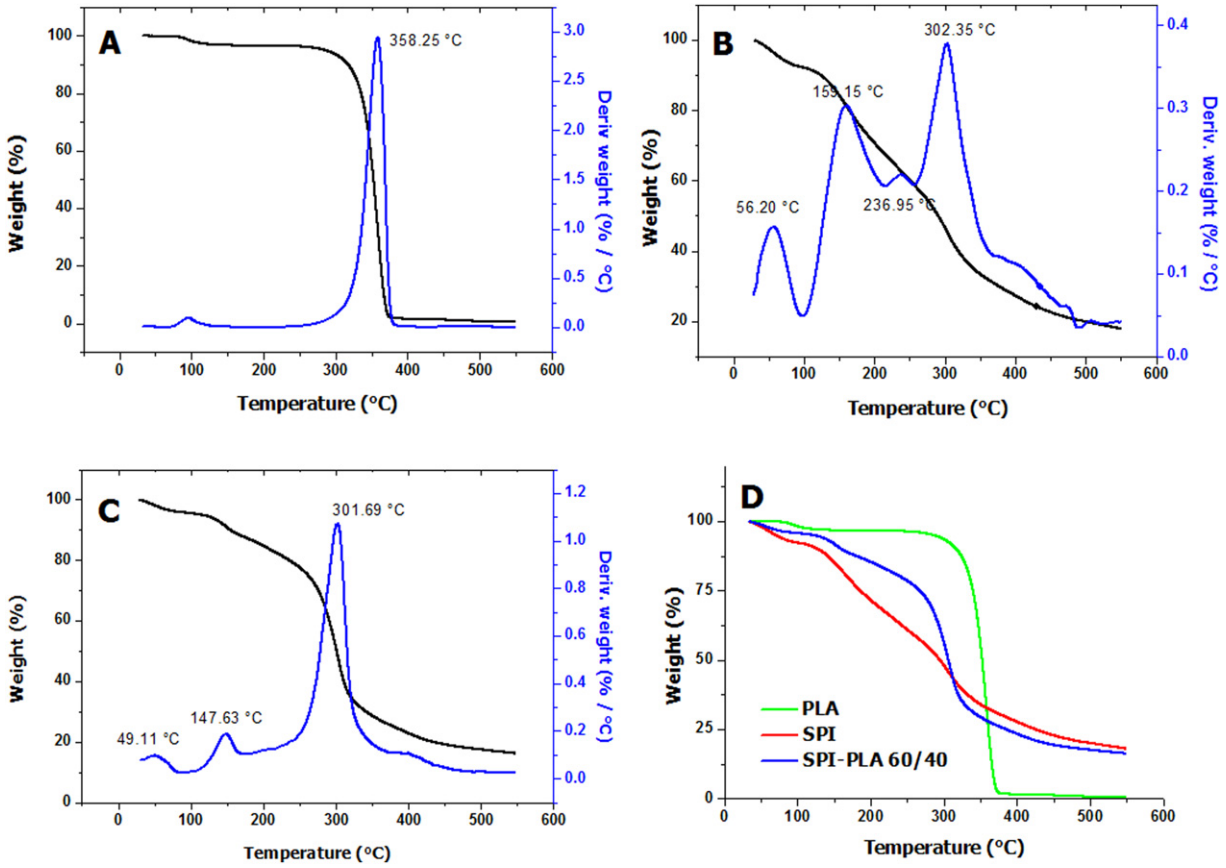

Fig. 1. TGA and DTGA (derivative) of the PLA (A); SPI 100(B); SPI–PLA 60/40 (C) films and overlay TGA of the three films (D).

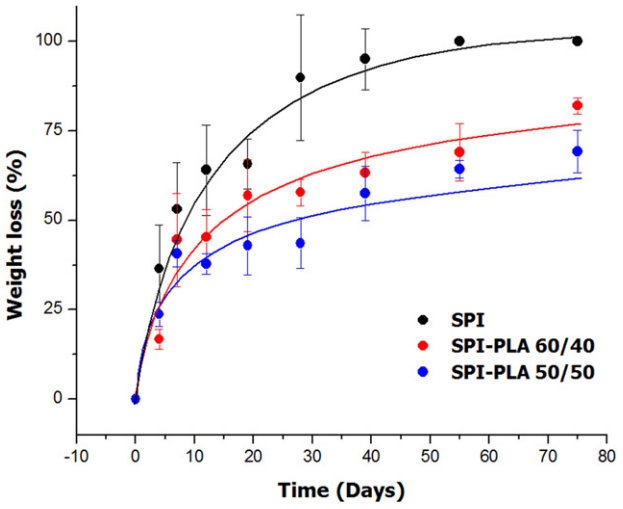

Fig. 2. Weight loss of the different films as a function of time.

turning into a more rigid material. A similar effect was described for the application of two PLA layers to a protein matrix (Martucci & Ruseckaite, 2010). The authors explain the fact that the mechanical response of the prepared films did not answer to a simple mixing rule is assigned to interactions between the components (the carbonyl group from PLA and the hydrogen from the peptide bonds in protein) in the protein/PLA interface.

3.6. Thermal properties

Thermal decomposition curves of the components used in film preparation and from the SPI–PLA 60/40 film were performed (Fig. 1). In the thermal decomposition curve of the SPI 100 film, four significant mass losses could be found. The first two (56.20 and 159.15 °C) correspond to the removal of water molecules present in SPI (included and adsorbed water respectively). Thermal decomposition of the protein involves a process beginning at 125 °C and ending at 350 °C, and two points of maximum speed (236.95 and 302.35 °C) can be seen for two different protein fraction degradations. At 550 °C, the residue was 18%. In the thermal decomposition curve of the PLA film, a drastic mass loss corresponding to the decomposition temperature of 358.25 °C of PLA could be observed. Moreover, the SPI–PLA bilayer film shows an overlap of the processes of mass loss in the components involved. In addition, loss of water as in the SPI 100 case can be found. Regarding the main degradation, it can be seen that this process shows a temperature similar to that of the SPI 100 film. In the three overlapping TGA, we can note that the bilayer film has better thermal behavior up to 300 °C (more stability) with respect to the SPI 100 film since, at equal temperatures, mass losses are lower. A similar thermal behavior for SPI–PLA blends is described in the literature (Calabria, Bandeira, Giacomelli, Filho, & Schmidt, 2009).

Differential scanning calorimetry curves were performed for the SPI, PLA and SPI–PLA 60/40 films. In the SPI curve, no melting temperature is found while in the PLA curve a melting point at 157.01 °C with a ΔHf of 18.70 J/g is observed. The curve of the bilayer film shows the properties of their components. In the bilayer films, the melting phenomenon is influenced by the SPI layer showing a decrease in melting temperature (154.48 °C) and ΔHf (6.328 J/g).

3.7. Biodegradation test

The degradation monitoring of the differently composed films (SPI 100, SPI–PLA 60/40 and SPI–PLA 50/50) was carried out for 75 days in soil burial conditions. The soil moisture was kept constant at (36 ± 3)%. This moisture value was kept below soil saturation to minimize mass loss by solubilization in water. The assay was performed in triplicate. The weight loss percentage in each film was represented in a graphic as a function of time (days). According to the data, it was observed that after 55 days, the SPI 100 film reached 100% of degradation while the 60/40 and 50/50 SPI–PLA films were degraded above 60 and 50%, respectively. These results suggest that the entire SPI layer was degraded while only a portion of PLA layer was degraded in the period analyzed. In a previous research work, a complete biodegradation of an SPI film was reached in 19 days using a similar biodegradation assay except that the assay was performed at a higher relative humidity (48 ± 4%) (González et al., 2011). This shows the close relationship existing between the

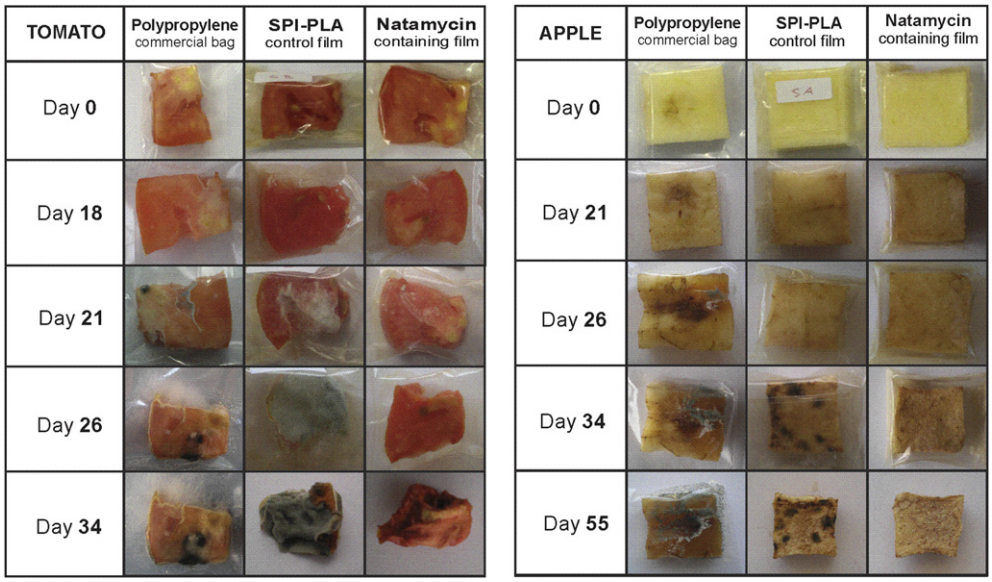

Fig. 3. Visual analysis of foodstuffs behavior coated with a commercial polypropylene, SPI–PLA 60/40 film without active agent and SPI–PLA 60/40 film containing natamycin.

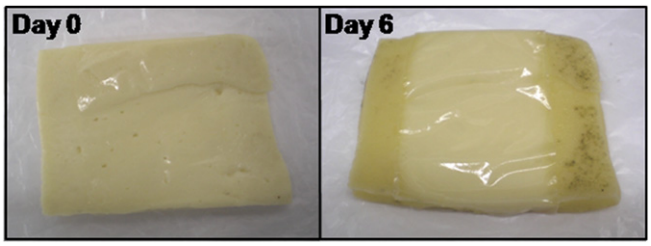

Fig. 4. Visual analysis of the behavior of a slice of soft cheese partially coated with natamycin-containing SPI–PLA 60/40 film before and after 6 days of storage.

available moisture and the soil microbial activity. After 75 days, degradation reached 80 and 65% for SPI–PLA 60/40 and 50/50, respectively. At this time, the PLA layer was partially degraded, showing a lowest degradation rate compared to the SPI component. The points in Fig. 2 show weight loss values (%) while solid lines indicate degradation tendency.

After being completed the physico-chemical characterization and degradation behavior, SPI–PLA films with active agents added were prepared to evaluate the performance of these materials as active food packaging.

3.8. Behavior on food

The behavior of the foodstuffs covered by the different films was visually evaluated in this assay. Three different materials were used as foodstuffs covering: a commercial polypropylene bag, SPI–PLA 60/40 film without active agent (control) and SPI–PLA 60/40 film containing natamycin as antifungal agent (0.33% w/w of SPI). The active agent was incorporated in the SPI layer since it acts more effectively than in the PLA layer. The behavior of the coated foodstuffs stored under similar conditions for all foods was studied for a maximum period of 55 days (Fig. 3).

The results obtained for tomato shows that on day 21, deterioration signs were observed with the appearance of mold in the tomato packaged with the SPI–PLA control film, and with the commercial polypropylene bag. These signs were not observed with natamycin-containing film. On days 26 and 34, the appearance of mold was more marked for all packages except for that containing natamycin. In this case, there was only a marked water loss, but with no mold found. Accordingly, it could be determined that the film-containing natamycin could delay the appearance of mold in the tomato for more than 13 days with respect to the polypropylene commercial bag. Analyzing the behavior of the coatings on apple, it could be seen that mold appeared in the commercial polypropylene bag on day 26; however, it was not found in other coatings. On day 34, the growth of mold could be noticed in the commercial packaging and in the SPI–PLA control film. With the active film, the apple only showed dehydration signs. The study in apple after 55 days concludes that the highest growth of mold was found in the commercial polypropylene packaging, and that the appearance of mold could be delayed for at least 29 days with respect to the polypropylene commercial bag using the natamycin containing film.

In addition, a slice of soft cheese was partially coated with the natamycin-containing bilayer film (SPI–PLA 60/40); an initial growth of mold on the free surface (not in contact with the coating) was observed after 6 days of storage at room temperature. By contrast, no presence of mold was found in the coated surface due to the inhibitory action of the active agent (Fig. 4).

3.9. In-vitro microbiological assays

Microbiological inhibitory assays of the films prepared with the active agents thymol (antibacterial) and natamycin (antifungal) on mold, yeast and bacteria (*E. coli* and *S. aureus*) were performed on Petri dishes using the agar disc diffusion method. Different quantities of antibacterial and antifungal agents were used; however, the best results were obtained with 0.33% w/w of natamycin and 25% w/w of thymol regarding SPI weight. As shown in Fig. 5, the antifungal action of natamycin was verified, since films containing this active agent inhibited the growth of mold and yeast. This result was obtained using a very low amount of natamycin (0.33% w/w of SPI). A small inhibition zone (about 2 cm in diameter) could also be observed. This suggests that the active agent diffuses through the film, producing inhibition of fungal flora in a region larger than that covered by the film. Determinations with films containing a higher amount of natamycin (0.52% w/w of SPI) showed a wider inhibition zone (results not shown). As expected, this film did not inhibit the growth of bacteria. A similar behavior on yeast and bacteria was also described for natamycin containing-whey protein isolate films (Ramos et al., 2012).

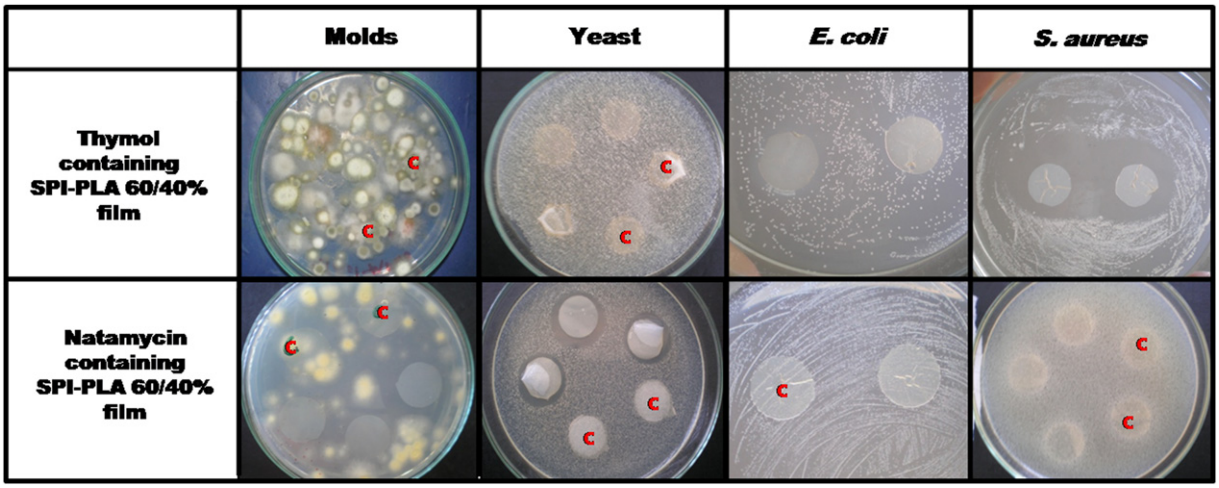

Fig. 5. *In-vitro* inhibition of mold, yeast and bacterial growth with thymol-containing SPI–PLA 60/40 and natamycin-containing SPI–PLA 60/40 films. C represents the control film (without active agent).

On the other hand, the films prepared with the addition of thymol inhibited the growth of the two bacterial strains (one Gram+ and other Gram−); yet, no inhibition of mold or yeast was observed. This assay concludes that the films prepared may be used to inhibit growth of different microorganisms on active packaging. Similar antibacterial agent-containing biodegradable films were previously described in the literature (Ahmad, Benjakul, Prodpran, & Agustini, 2012).

4. Conclusion

In this research work, biodegradable films were prepared without adding compatibilizing agents, adhesives, or without chemically modifying film surfaces. The films were physico-chemically characterized and the biodegradation behavior was studied. It was found that the addition of PLA markedly improved fundamental physical and mechanical properties of the films. The incorporation of low amounts of actives agents into the films was carried out showing positive results for mold, yeast and two strains of bacteria growth inhibition in an *in-vitro* microbiological assay. These results showed that these materials may be highly suitable as a biodegradable material for active food coatings.

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