Transglutaminase Crosslinked Gelatin Films Extracted from Tanned Leather Waste

by

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Abstract

The production of biodegradable polymers has proved to be a promising alternative, since these materials have accelerated degradation, contributing to the reduction of residues and the reduction of environmental pollution. The tannery wastes contain considerable collagen and can be used for gelatin extraction and film production for use in agriculture. Gelatin-based films, however, present some challenges for practical application, such as permeability and solubility in water, parameters that can be improved through the crosslinking process by employing enzymes, promoting the union of polymeric gelatin chains. In this context, the action of the enzyme transglutaminase was investigated to improve the properties of gelatin films recovered from leather and chitosan residues, which were evaluated according to thickness, solubility, permeability, mechanical properties, and soil degradation. The results indicated that the enzyme concentration in the films had a significant effect on the properties of water permeability and solubility and strain to rupture. The evaluation of soil degradation showed that films with higher enzyme addition took longer to be degraded.

Introduction

The polymers from non-renewable sources, despite the intense use in various sectors, such as for packaging production and agriculture, generate high volumes of waste, causing environmental impact due to the time they need to degrade. Biodegradable polymers become an alternative, since they are produced from renewable sources and have faster degradation kinetics, being considered environmentally correct.[1]

Brazil has the potential to use agro-industry waste, since large amounts of waste of animal and vegetal origin accumulate daily, causing problems with logistics and disposal of these materials.[2,3] Among the challenges related to technology to produce biodegradable materials, we highlight the reuse of hazardous waste, as the residues of chromium (III) tanned leather, generated by the tannery industries, which contain considerable protein content from animal hide collagen, being considered by the Brazilian standard ABNT NBR 10.004[4] as Class I - dangerous, due to their toxicity according to the leaching test ABNT NBR 10.005.[5]

There is a growing interest in the process of extracting collagen/gelatin and its derivatives due to the tendency to use this protein in place of synthetic compounds.[6] Gelatin is the product of denaturation and partial hydrolysis of collagen chains, which is an insoluble protein found especially in the skin and cartilage of cattle, pigs and fish, if used as a gelling agent for the development of polymeric films.[7] Gelatin stemming from residues of leather tanned with trivalent chromium has its applicability limited in the food area, due to the possible residual chromium, but promising for applications in agriculture.[8]

Gelatin-based films have good mechanical, optical and sensory properties, although they are sensitive to moisture and have high water vapor permeability due to their hydrophilic character. The ways to reduce the permeability and solubility in water of gelatin films is through the crosslinking process, which promotes the union of two or more polymeric chains.[9, 10, 11] The reticulation of gelatin by the enzyme transglutaminase can catalyze acyl group transfer reactions by forming intra and intermolecular crosslinks in proteins, peptides and various primary amines, mainly through covalent bonds between glutamine and lysine residues. This type of treatment, besides promoting a decrease in the hydrophilic character of gelatin films, also improves the mechanical properties of the material.[12]

In this context, the present work aimed to produce polymeric films using gelatin extracted from residues of chromium (III) tanned leather and to evaluate the effects of the transglutaminase enzyme (TGase) on the properties of thickness, permeability to water vapor, solubility, mechanical properties and degradation of the films in soil.

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Materials and Methods

Protein Extraction

Alkaline hydrolysis was used to extract gelatin from chromium (III) tanned leather shavings.[11] The leather shavings was supplied by a local tannery.

The extraction was performed in an orbital agitator (MA 832, Marconi, Brazil) for 6 h, at 70°C and 180 rpm using a ratio of 50 g of leather shavings, 250 ml of water and 2 g of magnesium oxide.[13] Gelatin in aqueous medium was separated from the solution by vacuum filtration. After filtration, gelatin was concentrated in a dialysis membrane (Polyflux®) with capillary configuration for protein concentration and salt removal.

Film Production

The films were produced with concentrated gelatin, glycerol was added as plasticizer, 20% (w/v) on the gelatin mass, with heating at a temperature of 60°C under agitation for 30 min. The enzyme transglutaminase (ACTIVA® YG, Brazil) was solubilized in water, at a concentration of 1 and 3% (w/v) on the gelatin mass. The gelatin solution temperature was reduced to 37°C, enzyme action time was 15 min. Chitosan was added in a concentration of 1% (v/v). Then, 90 mL of the solution was poured into Petri dishes (19.5 × 2.5 cm) and dried in an environment with controlled conditions (23 ± 2°C and 50% humidity of) for 48 h.

Physical Properties

Thickness

The thickness of the films was determined by means of a digital micrometer with a resolution of 0.001 mm (Mitutoyo, Japan). Ten measurements were performed at points located at the ends and in the center of the films and from this, the arithmetic mean expressed in mm was calculated.

Water Vapor Permeability

Water vapor permeability was determined according to ASTM E96-15[14] using the desiccant method. The films were sealed to the nozzle of a test bottle with a diameter of 2.5 cm containing 10 g of silica (4-8 mm) and these were arranged in an environment with humidity maintained at 75%. The weighing was performed every 1.5 h for a total of 11.5 h. The ratio between the variation of the assembly weight and the area of the bottle nozzle was compared with the elapsed time. The slope of the drawn line is the rate of transmission of water vapor. All weight measurements were performed using analytical balance (AUY 220, Shimadzu, Japan). Relative humidity (75%) was controlled using a saturated sodium chloride solution.

Solubility

The percentage of water-soluble material was determined according to the method described by Cuq et al., 1997[15] with some adaptations. Square samples of 2 × 2 cm were dried in an oven (Model A35ED, DeLeo, Brazil), at 70°C for 24 h. Subsequently, they were weighed on an analytical balance (Model AUY220, Shimadzu, Japan) to determine the initial mass. The dried samples were immersed in 50 mL of distilled water and kept under agitation for 24 h at 80 rpm and 25°C in a thermostatic bath (Model 501/1D, Nova Era, Brazil). After this period, the samples were removed from the water and dried for 24 h at 70°C and weighed to obtain the final mass.

Mechanical Properties

The determination of mechanical properties, maximum tensile strength and elongation at break, consists of an average of two repetitions. The tensile tests were performed on a universal machine (model DL 2000, Emic, Brazil). The films were cut into a standard rectangular shape (20 mm × 100 mm) according to the ASTM D882-12.[16]

Degradation of Films

The evaluation of microbiological susceptibility of the films was performed according to ASTM G160-03.[17] The samples were cut in the dimensions of 2 × 2 cm, in triplicate. The soil (40 g) used in this experiment was placed in transparent 200 ml polypropylene (PP) cups, the samples were incubated for up to 12 h. The samples of the films were taken at the times of 1, 3, 8 and 12 h and analyzed by Thermogravimetry and FTIR. Each sample was washed with distilled water.

Thermogravimetry

The films thermogravimetry was performed in a simultaneous thermal analysis instrument (Model Jupiter 449, Netzsch, Germany). Aliquots of 10 mg of the samples, were submitted to a heating rate of 10°C/min in nitrogen atmosphere (50 ml/min) from 20° to 700°C.

Fourrier Transform Infrared Spectroscopy

FTIR experiments were performed on an infrared spectrometer with Fourier Transform (Model Spectrum 400, Perkin Elmer). The equipment was operated in attenuated total reflection mode (ATR) using diamond crystal. Thirty-two scans were performed with a resolution of 2 cm^{-1} in the wavelength range of 450 to 4000 cm^{-1}.

Statistical Analysis

The statistical significance of the factors tested was evaluated by means of Variance Analysis (ANOVA). Statistical analysis was performed with the aid of Statistica Software 12 (StatSoft Inc.). A 95% confidence level was used in all statistical analyses.

Results and Discussion

Physical Properties

The results obtained for each physical property can be visualized in Figure 1. The values were also statistically analyzed to verify if the enzyme TGase had significant effect on the film's properties.

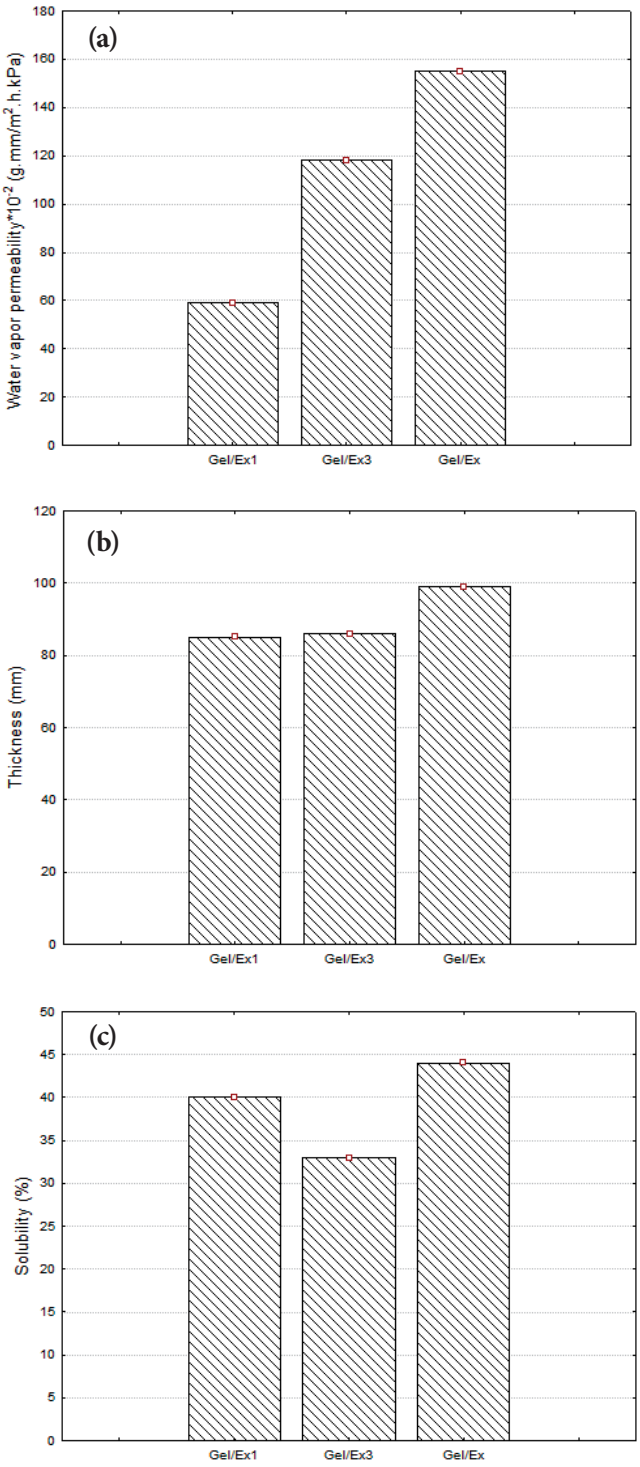

Figure 1. Physical properties of films **(a)** thickness, **(b)** water vapor permeability and **(c)** solubility. Gel/Ex1: Gelatin films with 1% TGase; Gel/Ex3: 3% TGase; Gel/Ex: no enzyme addition.

The statistical analysis indicated that the addition of enzyme had no significant effect on the thickness property (p = 0.72). The film without enzymatic treatment Gel/ Ex has a thickness of 0.099 mm and the films with enzymatic treatment, Gel/Ex1 and Gel/Ex3 have a thickness of 0.085 and 0.086 mm, respectively.

The different concentrations of enzyme showed a significant effect on the property of water vapor permeability (p = 5.05E-5). The permeability of the film without enzymatic treatment (Gel/Ex) was 1.55 g.mm.m^{-2}.h^{-1}.kPa^{-1}, and the films with enzymatic treatment Gel/Ex1 and Gel/Ex3 presented 0.59 and 1.18 g.mm.m^{-2}.h^{-1}.kPa^{-1}, respectively, that is, the enzyme at the concentration of 1 and 3% (w/w) promoted a reduction in permeability compared to the film without enzymatic treatment. This difference can be explained by the crosslinking process, which promotes the union of polymer chains through covalent bonds between the atoms. Thus, TGase reduces the space between the pores of the polymer matrix, making it difficult for the process of diffusion of water through the film.[12, 18] Similar results were observed by Wangtueai, Noomhorn and Regenstein, 2010,[19] who applied lizard-fish gelatin and obtained a permeability value of 26.3 g.mm.m^{-2}.d^{-1}.kPa^{-1} without adding enzyme to the film and, with 0.5% TGase, this value decreased to 21 g.mm.m^{-2}.d^{-1}.kPa^{-1}. The same was observed by Nishihora, Niehues and Quadri, 2015,[9] who worked with pig skin gelatin and obtained permeability values of 0.29 g.mm.m^{-2}.h^{-1}.kPa^{-1} for films without enzymatic treatment and, when adding 12.71 mg.ml^{-1} of TGase to the film, this value decreased to 0.1643 g.mm.m^{-2}.h^{-1}.kPa^{-1}. However, in a study carried out by Kołodziejska and Piotrowska, 2007[20], who used fish skin gelatin, the enzymatic crosslinking of the films did not allow improvement in the properties of barrier to water vapor, since the permeability of films not modified with TGase was 2.42 g.mm.m^{-2}.h^{-1}.kPa^{-1} and for those treated with 0.2 mg.ml^{-1} of TGase, this value was 2.40 g.mm.m^{-2}.h^{-1}.kPa^{-1}. It is highly likely that occurred because of different protein components of the films and the various enzymatic reaction conditions used in the preparation of the films.

On the other hand, when comparing the permeability values obtained in the films with the enzymatic treatment, Gel/Ex1 and Gel/Ex3, it's possible to observe that the increase in the concentration of TGase causes an increase in the permeability of the films, since the film with 1% of enzyme concentration obtained 0.59 g .mm.m^{-2}.h^{-1}.kPa^{-1} permeability and, by increasing this concentration by 3%, the permeability also increased by 1.18 g.mm.m^{-2}.h^{-1}.kPa^{-1}. The same was observed by Nishihora, 2015,[21] who worked with 60 mg.mL^{-1} of swine skin gelatin and 50 mg.mL^{-1} of glycerin as a plasticizing agent and, when adding enzyme in concentrations of 4 and 12 mg.mL^{-1}, obtained films with permeability of 0.7182 and 1.0328 g.mm.m^{-2}.h^{-1}.kPa^{-1}, respectively. This increase can be explained by the presence of plasticizer that, together with the increase in enzyme concentration, generates an increase in the free volume of the structure and, thus, facilitates the diffusion of moisture through the film. In addition, the higher concentration of TGase can cause an increase in the mobility of the chains, which results in a higher coefficient of water diffusion and, thus, greater water vapor permeability.[21, 22]

Statistical analysis indicated that the addition of the enzyme had a significant effect on solubility property (p = 0.023). The films obtained from gelatin without Gel/Ex enzymatic treatment showed solubility

of 44.37% and films with Gel/Ex1 and Gel/Ex3 treatment showed solubility of 40.38 and 33.12%, respectively. A decrease in solubility property occurs with increased enzyme concentration in films. This fact justifies the reduction of spaces in the polymer matrix, which occur due to cross-linking in the protein. Similar results were found by Yayli, Turhan and Saricaoglu, 2017,[23] when evaluating solubility in edible films derived from chicken gelatin treated with different concentrations of transglutaminase. The authors reported that there was a decrease in solubility as an increase in the concentration of TGase. Films treated with 1 and 3% enzyme obtained solubility values of 36.37% and 32.14%, respectively. Liu et al., 2017[24] studied bovine gelatin films treated with 0.6% TGase (m/m) and glycerol, dried at different temperatures. It was observed that as the drying temperature increased (from 15° to 35°C) there was a decrease in solubility in the films. The values for this property ranged from 36.7 to 34.4%.

Mechanical Properties

Gelatin films were characterized for mechanical properties, tensile strength at break and elongation. Figure 2 shows the results.

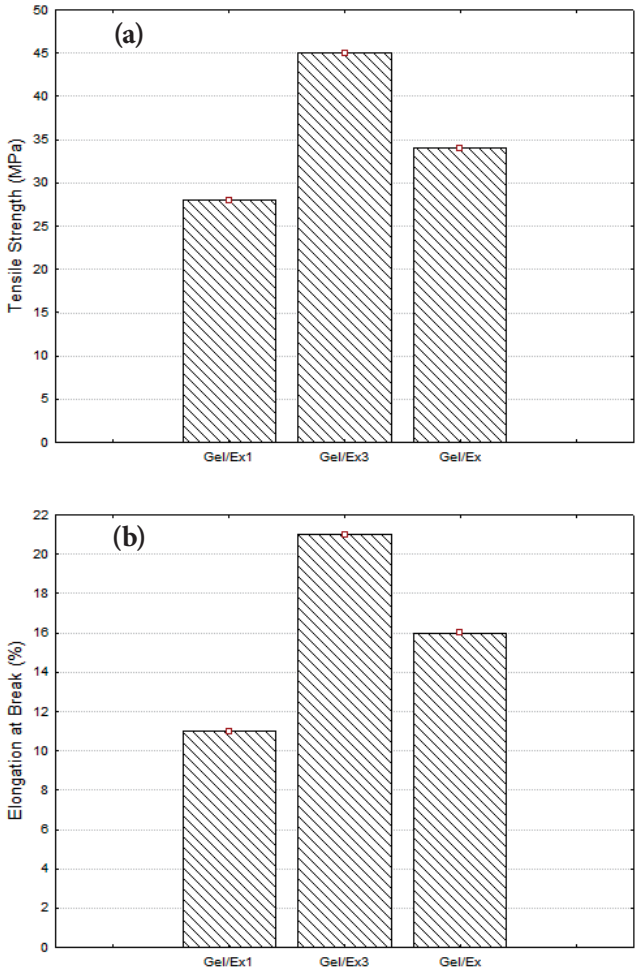

Figure 2. Mechanical properties of **(a)** tensile strength and **(b)** elongation at break of films obtained from recovered gelatin. Gel/Ex1: Gelatin films with 1% TGase; Gel/Ex3: 3% TGase; Gel/Ex: no enzyme addition.

The statistical analysis showed a significant difference for the tensile strength property of the films ($p = 0.028$), while for the elongation at break property, the statistical analysis did not present a significant value ($p = 0.109$).

The increase in the tensile strength for the film with the highest concentration of enzyme occurs due to the greater crosslinking between the gelatin molecules, acting as a reinforcement between the bonds of the matrix structure, configuring greater rigidity and less flexibility of the polymer.[18, 25] The mixture between chitosan and gelatin can also cause an increase in the mechanical properties due to interactions between electrostatic and hydrogen bonds, forming a more stable network between the polymers and, consequently, increasing the rigidity of this one.[26]

Masamba et al., 2016[27] analyzed zein films treated with different concentrations of transglutaminase and oleic acid. The concentration of 1% of TGase showed the highest value for tensile strength (26.9 MPa). Still, the authors found that low or high concentrations of enzyme in the formulation of the films can negatively affect the tension and elongation properties.

Jridi et al., 2014[28] and Hosseini et al., 2012[29] observed that films composed with fish gelatin and chitosan, plasticized with glycerol, had their tensile strength values increased as the chitosan content in the composition of the films increased. However, the values for elongation decreased as the presence of chitosan increased. The mixing of the two polymers caused the crosslinking of the polymeric matrix, where chitosan acted as a plasticizer, weakening hydrogen bonds and making the films more flexible.

A control film was produced with commercial gelatin, without the addition of an enzyme, which resulted in a tensile strength and elongation at break value of 65.5 MPa and 20.41%, respectively. The tensile strength is about 92% higher for commercial gelatin films when compared to gelatine recovered from residues, both without the addition of enzyme. It is observed that the addition of the enzyme has a positive effect, since when 3% of the enzyme is added to the formulation with extracted gelatin (Gel/Ex3), the rupture stress increases around 32% (34 MPa to 45 MPa), however the commercial gelatin film without addition of enzyme still has a tensile strength about 45% higher. The tensile strength in commercial gelatin films tends to be higher than in extracted gelatin films, since in the latter there is a greater presence of salts in their composition, which can cause the matrix to swell due to the hygroscopicity associated with minerals, thus causing an increase of the mobility of the polymer chains and weakening the bonding forces between them which, consequently, causes an increase in the deformation capacity and a decrease in the breaking force.[30]

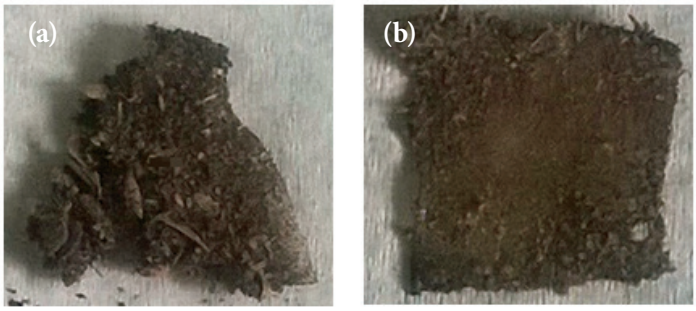

Figure 3. Films produced with gelatin extracted from chromium (III) tanned leather, with **(a)** 1% of TGase and **(b)** 3% of TGase, after 12 h of exposure into the soil

Films Degradation Assays

The evaluation of the microbiological susceptibility of the films was carried out according to the ASTM G160-03[17] standard.

In the present work it was not possible to determine the mass loss of the produced films, as the soil adhered to the material in the first hour of exposure and, consequently, the washing step for later weighing could not be performed. From Figure 3, the films are observed after 12 h of exposure into the soil.

The film without the addition of the enzyme transglutaminase (Gel/Ex) was already in an advanced stage of degradation after 12 h of exposure, and it was not possible to take photographs, as the degradation occurred before the first removal of samples from soil. The film with 1% of enzyme (Gel/Ex1) presented signs of degradation, while the film with 3% of transglutaminase enzyme (Gel / Ex3) was more intact. Thus, it was possible to observe that the increase in the enzymatic concentration gives greater integrity to the polymeric structure of the material, reducing the degradation when exposed to soil. Both films were completely degraded after 24 h of exposure to the soil.

In a study carried out by Lucena et al., 2017,[31] it was revealed that films produced with xylan and gelatin have high degradability (less than 15 days) and can be considered as a new raw material that is primarily non-polluting in industry.

Thermogravimetric Analysis of the Films Removed from Soil

For the Gel/Ex and Gel/Ex1 films, it was not possible to perform the thermogravimetric analysis, due to the soil adhered to the samples. Figure 4 reveals the thermogravimetry of Gel/Ex3 samples collected at different exposure times in soil.

The first stage of degradation is related to the beginning of the evaporation of more volatile compounds, such as free water present on the surface of the film. The second stage occurs due the decomposition of more complex molecules, such as polysaccharides and proteins, represented in these films by chitosan and gelatin, respectively. The last stage refers to the degradation of bonds still present in the polymeric matrix.[32]

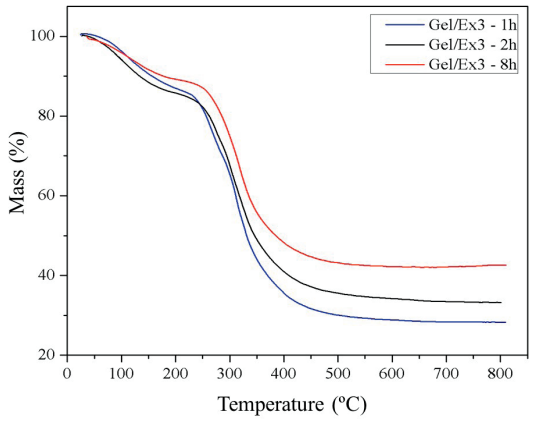

Figure 4. Thermogravimetry for films of extracted gelatin with 3% of enzyme (Gel/Ex3), after 1, 3 and 8 h of exposure in soil.

The residual mass content can be justified by two factors (1) because it is a film produced from gelatin extracted from leather residue, there may be the presence of salts from the tanning of the hides still attached to the protein molecule, and (2) the longer the exposure time to the soil, the ash content also increases, justified by the adhesion of organic and inorganic matter to the film and the formation of new products linked to the polymeric chain.[32]

FTIR from the Films Exposed to the Soil

Figures 5a and 5b show the FTIR spectra for films produced with gelatin extracted from chromium (III) tanned leather shavings, arranged in soil for the periods of 1 and 3 h.

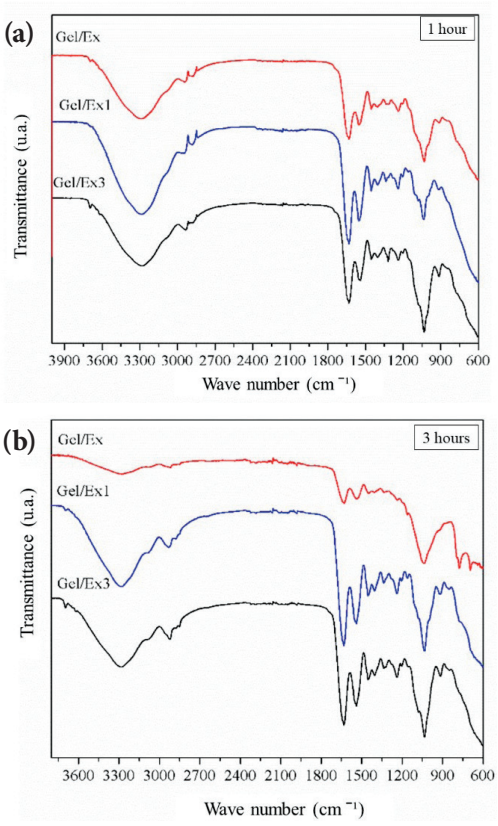

Figure 5. FTIR curves for extracted gelatin films (Gel/Ex, Gel/Ex1 and Gel/Ex3) after **(a)** 1 h exposure to soil and **(b)** 3 h exposure to soil. Gel/Ex1: Gelatin films with 1% TGase; Gel/Ex3: 3% TGase; Gel/Ex: no enzyme addition.

According to the FTIR graphs, it is observed that the peak corresponding to amide I (~ 1650 cm^{-1}) is reduced in intensity after 3 h of exposure to the soil (Figure 6b) for the film without enzymatic treatment (Gel/Ex). This peak represents the stretching of C = O (carbonyl) bonds that can be defined as an observation peak to check the degradation of a polymer due to the break in the bond between carbon and oxygen.

The decrease in vibrational bands for -OH (~ 3500 cm^{-1}) and -NH (~ 3600 and 1650 cm^{-1}) molecules indicates that there is a weakening of hydrogen bonds between the polymers that form the film due to the absence of an agent, such as the enzyme transglutaminase, which is able to stabilize chemical bonds between materials. The enzyme provides a stronger intermolecular interaction between the reactive groups and its absence in the films can cause the rupture of the polymeric network due to the action of external agents such as humidity and the action of microorganisms.[24, 29, 34, 35]

For the films with enzymatic treatment (Gel/ Ex1 and Gel/Ex3) it is noted that the peaks corresponding to the -OH stretch of water molecules and -NH stretch in wave number ranges between 3000-3600 cm^{-1} increase after three hours of exposure to soil. These observations indicate that the addition of transglutaminase enzyme interfered with the formation of hydrogen bonds between the -OH and -NH groups, demonstrating that there were changes at the molecular level when the enzyme and the polymer matrix interacted.[36] The absorption band present at 3300 cm^{-1} is due to the presence of glycerol -OH bonds that are expressed in this spectrum range, and to the absorption of water by the films, which have hygroscopic characteristics. In addition, the presence of glycerol can be confirmed by the presence of the peak close to 1080 cm^{-1}, typical of the interaction between the -OH group and gelatin through hydrogen bonds.[26]

In the range from 1235 to 1541 cm^{-1}, typical bands of gelatin samples are present, representing amine and amide groups (secondary and tertiary). The absorption band at 1541 cm^{-1}, frequently used in the identification of proteins, stands out.[37]

The FTIR analysis and the results obtained by visual analysis of the samples after three hours of exposure to the soil show that the enzyme was effective from the point of view of increasing the resistance to the degradation of the films, in addition to having contributed effectively to the increase of important mechanical properties.

Conclusion

Films produced from protein extracted from chromium III tanned leather residue had their properties improved with the addition of the enzyme TGase.

The tests indicated that the TGase acted in the films reducing the thickness, the amount of water vapor permeability and the solubility of the films. The mechanical properties such as elongation and rupture stress were increased for films with higher concentration of TGase enzyme. The degradation test in simulated soil proved that the films are biodegradable and that the addition of the enzyme transglutaminase promoted the crosslinking of the polymer matrix, maintaining the integrity and degradation of the films for a longer time.

Futhermore, biodegradable gelatin films are a promising alternative to materials produced from non-renewable sources. The films produced can be used as soil cover, providing essential nutrients, due to the presence of nitrogen in its polymer structure, in addition to contributing to the environment, with the reduction of the environmental impact caused by the slow decomposition of polymers.

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