Swelling, Mechanical, and Barrier Properties of Albedo-Based Films Prepared in the Presence of Phaseolin Cross-Linked or Not by Transglutaminase

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Edible films were obtained from *Citrus paradisi* grapefruit albedo homogenates and bean protein phaseolin modified or not by the enzyme transglutaminase. Swelling capability, barrier performance to water vapor, oxygen and carbon dioxide, and mechanical properties of such films were investigated. The addition of the protein, mostly in the presence of transglutaminase, provide films less swellable at pH values above 5 compared to films made by albedo homogenates only, whereas the action of the enzyme clearly improves mechanical properties producing more stretchable and elastic films. Moreover, transglutaminase-mediated cross-linking of phaseolin gives rise to films less permeable to carbon dioxide and able to offer a high barrier to water vapor. These findings suggest that albedo-phaseolin film prepared in the presence of transglutaminase can be a promising candidate to be used as food edible wrap.

Introduction

The development of packaging films based on biopolymers has attracted attention and renewed interest due to their environmentally friendly nature and their use in the food industry[1,2] with the aim to offer an alternative to plastic packaging material. The latter is widely applied in the food industry because of its low price and favorable functionality characteristics such as mechanical properties, good barrier properties to O_2 and aroma compounds, as well as heat sealability. On the contrary, plastics have a very low water vapor transmission rate and they are considered nonbiodegradable and therefore polluting for the environment. On the other hand, films made of natural polymers offer some advantages that depend on the nature of the component molecules. For example, protein films exhibit better oxygen barrier and mechanical properties than polysaccharide films. Thereby, two or more film-forming polymers of different nature can be used to prepare composite edible films. In the past few years we have developed many kinds of edible films using different polysaccharides and proteins.[3−7] For the polysaccharide component, we have used byproducts from different edible species, that is, *Foeniculum vulgaris* (fennel),[8] *Feijoa sellowiana* (feijoa),[9] *Fragaria vesca* (strawberry),[9] and *Prunus armeniaca* (apricot),[9] even though purified polysaccharides, such as pectins from *Malus communis* (apple)[3,4] and from *Citrus*,[7] have also been used. The film forming capability of such polysaccharides were tested in the presence of protein components of different origin previously modified or not by the enzyme transglutaminase.

Transglutaminase (TG, protein-glutamine:amine γ-glutamyl transferase. E.C. 2.3.2.13) catalyzes the formation of ε-(γ-glutamyl)-lysine cross-links into proteins via an acyl transfer reaction. The γ-carboxyamide group serves as the acyl donor and the ε-amino group of lysine serves as the acyl acceptor.[10] Moreover, reactive lysines may be substituted by several compounds containing a primary amino group, giving rise to a variety of protein-(γ-glutamyl) derivatives.[10,11] TG was successfully used to modify the biological activities of peptides and proteins by covalently linking polyamines to their reactive endoglutamine residues.[12−14] In addition, TG was employed for immobilizing enzymes,[15,16] as well as for improving the properties of several protein-based (such as casein, egg white, and whey proteins) edible films.[17−20] In the present study, we report the use of TG for producing films made of grapefruit (*Citrus paradisi*) albedo homogenates, as a source of polysaccharides, and phaseolin, a storage protein from *Phaseolus vulgaris*. Albedo is the inner part of the peel and represents a layer of white spongy material that directly protects the juicy part of the fruit. Albedo possesses a high pectin content,[21] thus, pectins used in the food industry as gelling and thickening agents are usually extracted from albedo of different species of *Citrus* (lemon, orange, grapefruit). Phaseolin is a protein that acts as a substrate for the enzyme TG.[22] Because of its globular nature, phaseolin has already been used successfully as a component of edible films.[22,23] Both albedo and phaseolin are abundant, renewable, and biodegradable materials, characteristics that make them attractive feedstocks for edible films and bioplastics. Here we report the preparation and characterization of films made in the presence of the enzyme TG with the aim of proposing them in food packaging.

Experimental Sections

Materials.

Phaseolus vulgaris L. beans and grapefruits (cultivar "Sweetie", Jaffa, Israel) were purchased from a local supermarket. Chemicals for electrophoresis were from Biorad (Segrate, Milano, Italy). Microbial TG (Activa WM), derived from the culture of *Streptoverticillium* sp., was supplied by Ajinomoto Co. (Japan). PEG and all other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Methods. *Film Forming Procedure.* The outer parts of albedo were cut into pieces of about 1 cm^3 in size, suspended in water at a concentration of 0.2 g mL^{-1} and heated at 95 °C for 30 min. The resulting material was treated using a kitchen juice extractor with a centrifugal filter having a cut off of 200 μm (Centricon, Ariete). The

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degree of esterification (68% ± 9.9) of pectins contained in the strained material was determined as described by Giosafatto et al.[23] This material was used for film preparation. Phaseolin was isolated from *Phaseolus vulgaris* beans by using the ascorbate-NaCl procedure described by Sun and Hall[24] and modified by Mariniello et al.[22] The protein, purified to homogeneity, was dissolved into distilled water at a concentration of 80 mg mL^{-1}.

Microbial TG was prepared by dissolving the commercial preparation in distilled water. The specific activity of the enzyme was 92 U/g. Estimation of enzymatic activity was carried out by a colorimetric hydroxamate assay according to Pasternack et al.[25] Prior to film casting, the solutions were degassed under vacuum to remove bubbles.

Three different kinds of films were prepared: albedo-based films (AH films), albedo-based films made in the presence of phaseolin (AH/Ph films) and albedo-based films made in the presence of TG-modified phaseolin (AH/Ph/TG films). Films were cast by pouring the solution into 5 cm diameter polystyrene Petri dishes. For AH film preparation, 15 mL of albedo solution were spread into the plates while, for AH/Ph films, albedo solutions (15 mL) were mixed with 2.4 mL of phaseolin solution and gently mixed. Finally, AH/Ph/TG films were obtained by adding 8.8 U of the enzyme to the final solution of albedo and phaseolin. All the samples were allowed to dry in a climate chamber at 37 °C and at 50% RH for 18 h. Dried films were peeled intact from the casting surface and conditioned at 50% RH and at 25 °C for 48 h before being tested.

Protein Determination. Protein determination was carried out by the Biorad Protein Assay (Biorad), using bovine serum albumin as standard.[26]

Film Characterization. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). A total of 20 mg of each film type were dissolved in 250 μL of sample buffer (15 mM Tris-HCl, pH 6.8, containing 0.5% w/v SDS, 2.5% v/v glycerol, 200 mM β-mercaptoethanol, and 0.003% w/v bromophenol blue), boiled for 5 min, and centrifuged for 10 min at 13000 × g. Aliquots of 20 μL of each sample supernatant were analyzed by 12% SDS-PAGE, as described by Laemmli.[27] Electrophoresis was performed at constant voltage (80 V for 2−3 h), and the proteins were stained with Coomassie Brilliant Blue R250. Biorad Precision Protein Standards were used as molecular weight markers.

Thickness. Film thickness was measured using an electronic digital micrometer with a sensitivity of ±2 μm (Metrocontrol, Srl, model HO62). Film strips were placed between the jaws of the micrometer and the gap was reduced until the minimum friction was measured. Mean thickness (mm) was determined from the average of measurements at five locations.

Scanning Electron Microscopy (SEM). SEM, used to characterize film surfaces, was used as described by Mariniello et al.[8] The samples were examined with a scanning transmission electron microscope (Philips Electronics, Mahwah, NJ, model X20). Three different samples of each type of film were subjected to SEM, and five different micrographs of each sample were taken. Micrographs were obtained at 400× magnification.

Film Water Vapor Permeability. Film water vapor permeability (WVP) was evaluated by a gravimetric test according to ASTM E96 (1993)[28] by means of a Fisher/Payne permeability cup (Carlo Erba, Italy) as described by Di Pierro et al.[4] Silica gel (3 g) was introduced into each cup, and a film sample disk with a diameter of about 6 cm was placed on top of the cup and sealed by means of a ring kept in place by three tight clamps. The film area exposed to vapor transmission was 10 cm^2. The assembled cups were weighted and then placed in a desiccator containing a saturated KCl solution that provided a constant water activity of 0.8434 at 25 °C. The desiccator was stored in a Heareus thermostatted incubator at 25.0 ± 0.1 °C. Cups were weighted at scheduled times and the water vapor transmission rate through the film was estimated by the linear portion of the diagram obtained by plotting the weight increment of the cup as a function of time. It was assumed that steady-state was reached once the regression analyses made by using the last four data points resulted in $r^2 \geq 0.998$.

The WVP was calculated from the equation

$$\mathrm{WVP} = X/(A\Delta p)\mathrm{d}m/\mathrm{d}t$$

where $\mathrm{d}m/\mathrm{d}t$ is the slope of the cup weight versus time once steady-state was reached, X is the film thickness, A is the film exposed area, and Δp is the water vapor pressure across the film. By assuming that the vapor pressure inside the cup, due to the presence of silica gel, can be taken as equal to zero, Δp becomes equal to the vapor pressure inside the desiccator given by the product of the water activity and water saturation pressure (P0) at 25 °C (P0 = 3.167 kPa).

CO_2 and O_2 Permeability. The carbon dioxide (CO_2) and oxygen (O_2) permeabilities were determined using a modification of ASTM Standard Method D 3985-81[29] with MultiPerm apparatus (Extrasolution s.r.l., Pisa, Italy). The samples, duplicates of each film, were conditioned for 2 days at 50% RH before measurement. Aluminum masks were used to reduce film test area to 5 cm^2. The testing was performed at 25 °C under 50% RH.

Swelling Experiments. Disks of freshly prepared films were cut out with a punch and bisected with a razor. The length of the straight edge was measured with a traveling microscope to 10 μm (PTI Liss, Hampshire, England). The polymer volume in the swollen state is a measure of fluid imbibed and retained by the film. The swelling behavior was evaluated as function of pH. In fact, different solutions were prepared in 0.4 MPa PEG, 20 mM NaCl, adjusted to the required pH with 0.1 M NaOH or 0.1 M HCl.

Film Mechanical Properties. Tensile strength and elongation to break were measured by using an Instron Universal Testing Instrument (Instron Engineering Corp., Canton, MA, model 5543A) according to ASTM (1997)[30] and following a procedure very similar to that described by Mariniello et al.[3] Film samples were cut into 10 mm wide and 100 mm length strips using a sharp razor blade. The strips were equilibrated overnight at 50 ± 5% RH and 23 ± 2 °C in an environmental chamber. Five samples of each film type were tested. Each film strip was placed between the pneumatic jaws of the Instron that were previously preset to give an original gauge of 90 mm and the strips were then stretched at a rate of 30 mm min^{-1} until sample failure. Measurements of the load (N) and deformation (mm) were used to calculate tensile strength (maximum load placed on the sample divided by the cross-sectional area) and the elongation to break (deformation of the sample at maximum load divided by original gauge length).

Statistical Analysis. JMP software 5.0 (SAS Campus Drive, Building S, Cary, NC) was used for all statistical analyses. The data were subjected to analysis of variance, and the means were compared using the Tukey-Kramer HSD test. Differences were considered to be significant at $p < 0.05$.

Results

Film-Forming Properties of Albedo Homogenates. In the present paper, albedo from grapefruit of brand Jaffa, cv. Sweetie (Israel) was used, even though no differences were observed when common grapefruits were used. The procedure used to extract polysaccharides from albedo homogenates gave rise to a pectin solution with a methyl esterification degree of 68% ± 9.9. Films were prepared by solution casting in the presence of phaseolin, extracted from common beans, as protein component. TG was added as cross-linking enzyme in some pectin albedo-phaseolin formulations because phaseolin was shown to be able to act as an effective substrate for such enzyme.[22] In particular, pectin solutions from albedo homogenates, both with and without protein and enzyme components, were stratified into polystyrene Petri dishes and dried under appropriate conditions of temperature and humidity. After 18 h it was possible to obtain

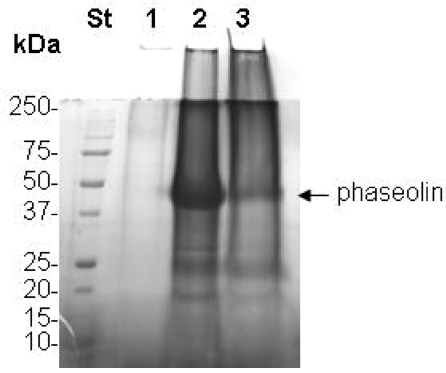

Figure 1. SDS-PAGE of films followed by Coomassie staining: lane 1, AH films; lane 2, AH/Ph films; lane 3, AH/Ph/TG. Further experimental details are given in the text.

Table 1. Water Vapor Permeability of AH, AH/Ph, and AH/Ph/TG Films[a]

	thickness (mm)	WVP (cm^3 mm m^{-2} day^{-1} kPa^{-1})
AH	187 ± 6.1	11.64 ± 0.81
AH/Ph	253 ± 4.5	16.62 ± 1.05[b]
AH/Ph/TG	256 ± 5.5	2.71 ± 0.30[c]

[a] The results are expressed as means of 10 replicates ± standard deviation. Further details are given in the text. [b] AH/Ph vs AH, significance, $p < 0.05$. [c] AH/Ph/TG vs AH/Ph, significance, $p < 0.001$.

three different films: AH, AH/Ph, and AH/Ph/TG films. All films macroscopically appeared yellowish, handled, and flexible and were easily removable from the plates. The SEM analysis of such films demonstrated that the distribution of the two components was homogeneous, with a smoother surface when the enzyme was present as cross-linking agent (data not shown). Similar results were obtained by Mariniello et al.,[8] studying films made of fennel homogenates and phaseolin modified or not by TG.

To investigate whether TG leads to polymer formation even in the presence of albedo homogenates, the three kinds of films were solubilized and analyzed by SDS-PAGE (12%) (Figure 1). The obtained results suggest that the TG reaction carried out in the casting system gives rise to phaseolin polymerization. In fact, the intensity of phaseolin monomer band (lane 2) was observed to decrease in the presence of the enzyme (lane 3).

Film Barrier Properties. *Water Vapor Permeability (WVP).* There are some limitations to the application of polysaccharides and proteins from vegetal origin for food packaging, because of their high sensitivity to moisture. In this paper we exploited the strategy to overcome this drawback by reticulating enzymatically the protein component of albedo-based hydrocolloid films by means of TG. As it is possible to observe from Table 1, the films prepared in the presence of TG show a strongly reduced WVP compared to films in which the cross-linking agent is absent. In fact, in the absence of the enzyme, phaseolin causes a rise of film WVP compared to the one exhibited by the film prepared only with albedo homogenates.

CO_2 and O_2 Permeability. To use an edible film for food packaging, it is of great importance to study its permeability to gases such as CO_2 and O_2. The gas permeability was measured at a RH of 50% and a temperature of 25 °C. From the results shown in Figure 2 it is clear that when phaseolin is added to the albedo homogenate there is a significant reduction of CO_2 permeability and this reduction is even more evident for the films prepared with samples into which TG was included in the mixture. Concerning the analysis of O_2 permeability, the presence of the protein provides a film a little more permeable

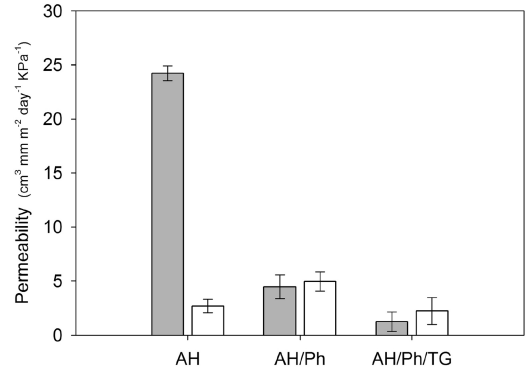

Figure 2. Carbon dioxide (gray bars) and oxygen (white bars) permeability of AH, AH/Ph, and AH/Ph/TG films. The results are expressed as means of five replicates ± standard deviation. Further experimental details are given in the text.

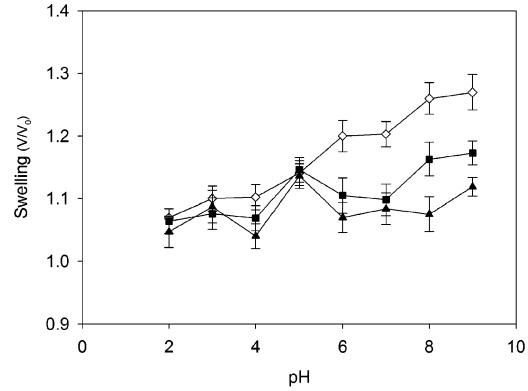

Figure 3. Swelling dependence of pH at 0.4 MPa osmotic pressure and 20 mM NaCl of AH (◇), AH/Ph (■) films. The results are expressed as means of five replicates ▲ standard deviation. Further experimental details are given in the text.

to O_2, while the addition of the cross-linking enzyme gives rise to a film with an O_2 barrier property, similar to the one exhibited by the film containing only AH.

Film Swelling. To investigate the swelling behavior of the three different types of films prepared, we performed swelling experiments as function of pH. It is possible to observe from Figure 3 that films obtained only from albedo homogenates show a higher degree of swelling at pH values higher than 5. This could be due to the fact that pH values higher than 5 cause an increase in ionized carboxyl groups of albedo pectins, with a consequent repulsion that is responsible for the swelling increase. The addition of phaseolin gives rise to a film that swells in a minor degree because of the amphoteric nature of the protein, as previously reported by Giosafatto et al.[23] The presence of TG, provoking the formation of isopeptide bonds between some glutamine and lysine residues occurring in phaseolin, gives rise to a film that shows a swelling behavior similar to the one exhibited by the film containing un-cross-linked protein. At alkaline pH values, the AH/Ph/TG film swells barely less than AH/Ph film, probably for the stabilizing effect of the enzyme over the structure of the film. Similar results were obtained by Di Pierro et al.[6] with films made with chitosan and ovalbumin.

Film Mechanical Properties. Film mechanical properties were evaluated by measuring the tensile strength and the elongation to break, which is an index of film extensibility. Figure 4 shows that the films prepared in the presence of the enzymatic cross-linking agent exhibit a % of extensibility significantly greater than the one shown by AH and AH/Ph

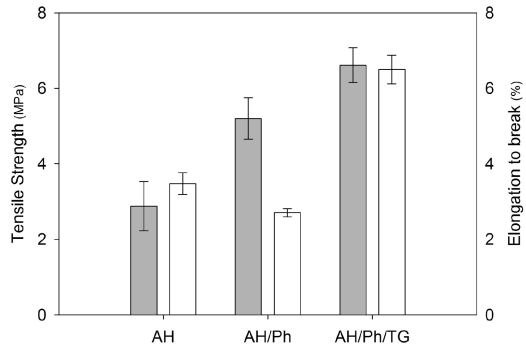

Figure 4. Film tensile strength (gray bars) and elongation to break (white bars) of AH, AH/Ph, and AH/Ph/TG. The results are expressed as means of five replicates ± standard deviation. Further experimental details are given in the text.

films. On the other hand, tensile strength, which is the maximum tensile stress a film could sustain, increases in the presence of phaseolin and even more in the presence of TG-modified phaseolin.

Discussion

In the present paper we analyzed the film forming properties of albedo obtained from the cultivar "Sweetie" of *Citrus paradisi* (grapefruit). In the attempt of finding an alternative way to treat albedo, which is mainly used to extract pectins for the food industry, we used it as polysaccharide component for our hydrocolloid films prepared also in the presence of phaseolin, a protein extracted from *Phaseolus vulgaris*, modified or not by TG. First, we demonstrated that grapefruit albedo itself possesses film forming properties, as it has been reported with orange albedo when it was used to prepare films cast in the presence of pectins, starch, and glycerol.[31] Then, we showed that phaseolin is cross-linked by the enzyme even in the presence of albedo, confirming the capability of this protein to act as both acyl donor and acyl acceptor substrate of microbial TG.[22] Thus, under film-casting conditions, the TG-mediated cross-linking reaction was shown to occur even in the presence of the polysaccharide component, which does not interfere with the formation of isopeptide bonds, giving rise to phaseolin polymerization. Similar results were previously obtained by Mariniello et al.[3] who demonstrated that purified commercialized pectins promote the TG-mediated polymerization of soy bean flour proteins in films made of pectin-soy flour and cast in the presence of the enzyme.

The presence of the protein component greatly influences the properties of albedo-based films. In particular, we have investigated the swelling properties dependence on pH using both osmotic pressure (0.4 MPa) and constant salt concentration (20 mM). For weakly charged polyelectrolytes, like pectins occurring in albedo homogenates, an increase of $[H^+]$ reduces the number of ionized carboxyl groups, consequently reducing swelling. When the degree of ionization increases, the electrostatic repulsions among the chains provoke an extended hydrophilicity of the network that is responsible for greater swelling ratios.[32] The presence of phaseolin, especially when the protein is cross-linked by the enzyme, reduces the film capability to swell probably because of the formation of electrostatic interactions between polysaccharide and protein components. In fact, as it is well-known, the degree of swelling of a polymeric material strongly depends on the amount and nature of the intermolecular chain association.[33] Similar results were obtained by Di Pierro et al.[6] using chitosan and ovalbumin as edible film components. Thus, the use of the protein (modified or not by the enzyme) can give rise to films exhibiting a limited response to moisture, which is an important property for assessing the application of such films.

Table 2. Permeability Properties of AH/Ph/TG Films and of Commercially Available Petrochemical-Based Plastics

film	WVP	PO_2	PCO_2
	$cm^3\ mm\ m^{-2}\ day^{-1}\ kPa^{-1}$		
AH/Ph/TG	2.71 ± 0.30	2.21 ± 1.25	1.23 ± 0.67
LDPE	0.50[a]	1.87[b]	
PVC	0.00613[c]	0.0078−1.554[c]	0.11−2.33[c]

[a] See ref 40. [b] See ref 38. [c] See ref 39.

Furthermore, TG-mediated cross-linking is also able to influence film WVP. It is well-known that both polysaccharide- and protein-based films offer a poor barrier to water vapor due to their hydrophilic nature. In our previous reports, recently summarized by Porta et al.,[34] we have observed that the reticulation of the protein component by means of the enzyme always leads to a reduction in the transmission of water vapor through the film. The addition of un-cross-linked phaseolin to the albedo-derived matrix increased the WVP of these materials, while films prepared in the presence of both phaseolin and TG showed lower permeability to water vapor. The increase in WVP of the AH/Ph films could be directly associated to the hydrophilic nature of the protein.[35] Conversely, the lower WVP shown by films made in the presence of TG should be due to the changes in the hydrophilic properties of the films together with the disappearance of primary amino and amide groups and the formation of less hydrophilic secondary amide linkages. Similar results were previously obtained in our laboratories using, as the polysaccharide component, purees from different fruits[9] or vegetables,[8] as well as purified carbohydrates such as chitosan or pectins with different kinds of proteins.[5−7]

In the same manner, albedo films containing TG-mediated reticulated phaseolin exhibit improved gas barrier, especially to CO_2. In the film made of only AH the degree of transmission of CO_2 is very high, while the same film acts as a good barrier to O_2. These results are due to the fact that CO_2 (which is a polar molecule) can easily be transmitted through the AH film made of pectin, a polymer that is charged itself. For the same reason, the AH film offers a high barrier to O_2 because this molecule is not polar. The presence of phaseolin in the film provokes a decrease in the available charged groups,[36] with the consequence that the transmission of CO_2 is negatively affected, while the transmission of O_2 increases.[37] When phaseolin is cross-linked by TG, both the degree of ionization and the free volume decrease, giving rise to a film with reduced permeability to both gases. A similar behavior was exhibited by other edible films, previously obtained in our laboratories in the presence of TG, using as components different polysaccharides and proteins of various origins.[4,34]

Finally, TG was proved to influence mechanical properties of albedo-phaseolin films. The presence of TG-mediated isopeptide cross-links was shown to improve the film capability to stretch, while the increase in the elongation to break values demonstrate that TG provides films more elastic.

These mechanical properties, together with the changed permeability features suggest an use of AH/Ph/TG films to wrap foods in substitution of the plastic-based ones. With this purpose we compared our novel Bioplastic with some widely employed commercial materials (see Table 2). Regarding to O_2, our Bioplastic exhibits permeability values similar to the ones

reported for LDPE,[38] while AH/Ph/TG films offer a poorer barrier compared to PVC-based plastics.[39] CO_2 permeability values of AH/Ph/TG films are comparable to PVC, whereas WVP of AH/Ph/TG films is 4 times higher than the values exhibited by LDPE[40] and 500 times higher than the PVC one. Plastic is the most widely used food wrap, but water vapor commonly condenses on the inner surface of plastic packaging materials, leading to potential microbial contamination of fresh products.[41] Thus, a film providing a moderate barrier to water vapor is desirable, because a high WVP is not advisible, as it can result in excessive loss of moisture during storage. To this regard, AH/Ph/TG could represent a valid candidate to replace petrochemical-based plastic packaging materials with the double aim to reduce pollution and recycle high value byproducts such as albedo contained in the *Citrus* sp.

Conclusions

Albedo homogenates were proved to have film forming properties due to their high pectin content. Transglutaminase cross-linking of phaseolin, an effective substrate of the enzyme, was still achieved in the presence of albedo-derived polysaccharide component during film preparation by casting. The enzyme-catalyzed isopeptide bonds were able to influence swelling, mechanical and barrier properties of the albedo/phaseolin-based films suggesting their possible employment as food wrapping.

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