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Hydrolytic Stability of L-(+)-Ascorbic Acid in Low Methoxyl Pectin Films with Potential Antioxidant Activity at Food Interfaces

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Abstract L-(+)-Ascorbic acid (AA) was compartmentalized into a low methoxyl pectin (LMP) film in view of localized antioxidant activity at food interfaces. The AA hydrolysis was specifically studied in the present work in order to determine the ability of the formulated LMP film to stabilize AA. Hence, films were stored at controlled relative humidity (RH) in the absence of air. A commercial LMP characterized by a 40% degree of methylesterification (DM) was used. Since sucrose is normally added for its standardization, films were also made with the dialyzed LMP in order to determine the sucrose effect. Glycerol was used for plasticization. Kinetics of AA loss and subsequent browning development were determined, which are dependent on the RH. Considerable AA retention ($t_{1/2}$=744, 727, and 185 days) was achieved at 33.3%, 57.7%, or 75.2% RH, respectively, at 25 °C. Browning rate constants decreased in one order of magnitude with respect to kinetic constants determined from films previously developed with high methoxyl pectin (HMP; DM of 73%). Absence of sucrose in the LMP network only affected the browning kinetics at 75.2% RH. The glass transition temperature (T_g) decreased with the increment of moisture content of the films and in a similar degree ($T_g \approx -90$ °C) to that observed for the HMP films, indicating the contribution of water to the network plasticization. However, water was more confined in the LMP network as inferred from the water availability determined by the ^{1}H-NMR and DSC. This was attributed to the water interaction at the Ca^{2+} junction zones. Sucrose seemed to hinder the retention of water molecules by the polymeric network at 75.2% RH.

Keywords Edible film · Ascorbic acid · Pectins · Browning · Sucrose · Water

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Introduction

Consumers are aware of the importance of a healthy diet, and at the same time, they request easy-prepared foods from the industry. The food industry shows an increased interest in product innovation in order to satisfy consumer's demand for high-quality and a variety of options of healthy products. For example, functional foods include foods fortified with health-promoting additives like natural antioxidants and vitamin-enriched products which bring about specific health benefit while satisfying the appetite and consumer sensory preferences (Redgwell et al. 2008).

Additives used in healthier food formulations (antimicrobial agents, antioxidants, etc.) should be as natural as possible. As

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well, additives must be used at the lowest but most effective concentration level. A localized activity of preservatives could be the way to achieve the latter, and edible biopolymer films are effective for carrying additives with localized activity at food interfaces. Compartmentalization into an edible film can overcome negative interactions between preservatives used in a food system or between a preservative and the nutrients in food formulations. L-(+)-Ascorbic acid (AA) is a reducing agent and also a water-soluble antioxidant for food preservation. In recent years, there has been an enormous demand for natural antioxidants mainly because of adverse toxicological reports on many synthetic compounds (Miková 2001). The importance of antioxidants contained in foods is well appreciated in food preservation and supplying essential antioxidants in vivo (Shi 2001). AA stability is affected by processing and storage conditions because it depends on a large number of factors such as temperature, equilibrium relative humidity (RH), oxygen partial pressure, light, package permeability, and package configuration (Kitts 1997). Furthermore, AA could be lost due to different factors such as oxidation in the presence of oxygen, metal ion catalysis, and the exposure to light (Bissett and Berry 1975). Anaerobic degradation also proceeds simultaneously when oxygen is present, but slower as indicated by Kurata and Sakurai (1967). On the other hand, non-enzymic browning (NEB) is related to AA loss since the products of the reactions that follow the first step of AA destruction are also part of the NEB reaction chain (Rojas and Gerschenson 2001). Therefore, a film network carrying AA might help its stabilization because AA compartmentalization can preclude its interaction with food preservatives or nutrient components; it can also provide a localized antioxidant activity at food interfaces.

Pectins are polysaccharides present in the primary cell walls and middle lamella of plants. Hence, these biodegradable polymers are also renewable (Bélafi-Bakó et al. 2011). They are rich in galacturonic acid (GalA) and often contain significant amounts of rhamnose (Rha), arabinose (Ara), and galactose (Gal) as well as 13 other different monosaccharides (Vincken et al. 2003). Three major pectic polysaccharide domains are recognized: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (Willats et al. 2006). Both the degree of methylesterification (DM) and acetylation (DA) have profound effects on functional properties (Willats et al. 2001; Guillotin et al. 2005). Commercial pectins are mainly constituted by HG where lateral substitution of the RG-I kinks has been partially hydrolyzed during the extraction process (Voragen et al. 2009). As other biopolymers, pectins are able to form continuous crystalline and/or amorphous microstructures like films. These network characteristics also depend on the preparation conditions. Plasticizers are chemically compatible small molecules used in film formulation which produce a molecularly miscible mixture that prevents the only interaction between macromolecules, leading to flexible films. Hence, their glass transition temperature (T_g) is considerably lower than that showed by the biopolymer alone and decreases as the plasticizer concentration increases (Kasapis 2008). The film formation mechanisms of biopolymers include intermacromolecular physical bonds such as electrostatic, hydrophobic interactions, and hydrogen bonds. Hence, filmmaking conditions have to be exhaustively controlled because any changes could alter the kinetics and mechanisms involved in the development of this kind of film networks.

In the present work, AAwas compartmentalized into a LMP film as a way of achieving localized antioxidant activity at food interfaces. The AA hydrolysis was specifically studied in order to determine the ability of the formulated LMP film to stabilize AA. Therefore, films were stored at controlled RH (33.3%, 57.7%, and 75.2%) in the absence of air. Pectin was selected in the present work by considering that the alternating presence of "disorder" (hairy) regions together with ordered (HG) ones could immobilize water sufficiently in the film microstructure for better AA retention (León and Rojas 2007). A commercial LMP (DM=40%) was used. Since sucrose is normally included for standardization of this pectin, films were also made using the dialyzed LMP. Calcium was added in the film formulation for gelling. The effect of the DM of pectins on the AA stability and subsequent browning development was analyzed by comparing with results previously obtained from HMP films. The effect of sucrose presence was also stated.

Materials and Methods

Chemicals

Food grade pectin with a low degree of methylation (GENU™ pectin type LM-12 CG) for manufacturing foodstuffs was from CP Kelco (J. M. Huber Corporation, Edison, NJ, USA). It was used either directly or after exhaustive dialysis against deionized water for 24 h followed by freeze-drying, in order to eliminate sucrose added by the manufacturer for standardization. The composition of the dialyzed LMP was determined, and results are shown in Table 1. The sucrose content of the commercial or food grade form was 32.0% *w/w*. All other chemicals were of analytical grade from Merck (Buenos Aires, Argentina) or Sigma–Aldrich Chemicals (St. Louis, MO, USA).

Experiments

Chemical Analyses of Pectin

Deionized (Milli-Q™) water was used for the preparation of all reagents. The chemical analyses performed according to Fissore et al. (2010) were:

Table 1 Molecular composition of the low methoxyl pectin used that was determined after dialysis of the CP Kelco™ pectin

Molecular weight ($\times 10^3$ Da)[a]	Total carbohydrate content[a,b,c] (%)	Galacturonic acid[a,b] (%)	Degree of methylation[a,b,d] (%)		Degree of acetylation[a,b,d] (%)	
734.2	99±3	85±2	40±3		6.0±0.6	
Neutral sugar composition[a] (mol/100 mol)						
Rha	Fuc	Ara	Xyl	Man	Gal	Glc
39.3	0	5.9	0	0	46.9	7.9
Cation content[e] (mol/g)						
Na	K		Ca		Mg	
2.41×10^{-4}	1.33×10^{-5}		6.15×10^{-5}		7.66×10^{-6}	

Composition showed by the low methoxyl pectin after dialysis through a membrane with a molecular weight cutoff of 12,000 (Sigma, St. Louis, USA)

[a] A mean molecular weight is shown. Chemical assays were performed as indicated under the Experiments section

[b] Mean and standard deviation (n=3) are shown

[c] Total carbohydrates are expressed as grams of D-galacturonic acid (standard for calibration curve) per 100 g of product (Fissore et al. 2010)

[d] Degree of methylation was calculated as the molar ratio with respect to D-galacturonic acid content, whereas degree of acetylation was the molar ratio with respect to the total carbohydrate content

[e] Cation content was determined through atomic absorption spectrometry (Fissore et al. 2009)

- Uronic acids, using D-galacturonic acid (GalA) for the calibration curve.
- Total carbohydrates, using GalA for the calibration curve.
- Methanol content.
- Acetyl groups.
- Sugar analysis: The proportions of neutral monosaccharides were determined after hydrolysis with 2 M-CF_3COOH (trifluoroacetic acid, TFA) for 2 h at 121 °C, followed by derivatization to the alditol acetates, and analyzed by gas–liquid chromatography (GLC) coupled to flame ionization detection for quantitative determinations or to electron impact-mass spectrometric detection when qualitative characterization was required. The GLC–mass spectrometry (MS) analysis of the alditol acetates was carried out to confirm structures of unusual sugars present in RG-II in a Shimadzu QP 5050 A GC/MS apparatus (Japan) working at 70 eV and using He as the carrier gas. Chromatographic runs were isothermally performed at 220 °C.
- Carboxyl reduction of the sample with a soluble carbodiimide: The proportions of neutral monosaccharides (galactose from polygalacturonic or HG chains) obtained after GalA reduction were again determined after hydrolysis with 2 M-CF3COOH (TFA) for 2 h at 121 °C using inositol as an internal standard. Hydrolyzates were derivatized to the alditol acetates and analyzed by GC as previously indicated.

The DM in percentage was calculated as the molar ratio between the methanol content and GalA in a given sample, whereas the DA was calculated as the molar (percentage) ratio between the acetyl content and the carbohydrates in the sample.

All chemical analyses were performed in triplicate.

Molecular Weight Profile

Samples were dialyzed against 0.5-M imidazole buffer (pH 7.0) at 5 °C during 48 h before freeze-drying after immersion in liquid nitrogen. The solid obtained was then dissolved in the 0.5-M imidazole buffer, as recommended by Mort et al. (1991). Molecular weight profiles were determined by gel filtration analysis using a fast protein liquid chromatograph (Pharmacia, Sweden) with a Superose 12HR 10/30 column (Amersham Biosciences–GE Healthcare, Buenos Aires, Argentina), eluted with 0.5-mol/L imidazole buffer (pH 7.0) at 0.5 ml/min (Wessels et al. 1998). Dextrans of 65,000 and 40,210 Da of molecular weights and blue dextran (Sigma, St. Louis, MO, USA), $CoCl_2$, and sucrose (Merck, Argentina) were used as standards for column calibration. Total carbohydrate content was determined into each collected fraction through the phenol–sulphuric acid spectrophotometric method (Dubois et al. 1956).

Filmmaking Procedure

The films were made using the casting technology. An aliquot of 5.25 g of dialyzed and freeze-dried LMP powder or 8.00 g of the commercial one was slowly poured in deionized water. This solution was continuously stirred under controlled high-speed shear (1,400 rpm-constant) in a vertical stirrer (model LH, Velp Scientifica, Italy) in order to

reach homogeneous hydration of the powder without lumps. While stirring, the obtained viscous, homogeneous, and transparent system was then heated at a constant heating speed (5.0 °C/min) on a hot plate (Velp Scientifica, Italy), with simultaneous recording of the temperature using a thermocouple connected to a Consort millivoltmeter (P 901, CE Belgium). Glycerol was added for plasticization, followed by potassium sorbate (0.03% *w*/*w*), AA (0.100% *w*/*w*), and $CaCl_2 \cdot 2H_2O$. The total weight of the system was completed by adding enough deionized water, followed by stirring for homogenization. The pH of the film-forming solution was 2.93. This hot solution was placed under vacuum for 20 s to remove air bubbles and immediately poured onto leveled polystyrene plates. The fractionated system was dried in a convection oven of controlled air speed for 2.5 h at a constant temperature. The films were peeled from the polystyrene plates and stored in desiccators over saturated solutions of known water activity (a_W^o) (Favetto et al. 1983; Greenspan 1977), in order to maintain a constant relative humidity (a_W=RH%/100) for film equilibration. The salts used were $MgCl_2$ (a_W^o=0.333), NaBr (a_W^o=0.577), and NaCl (a_W^o=0.752) at 25 °C. Equilibration was assessed by the measurement of a_W of the film samples every day until attaining the same value of RH%/100, corresponding to a_W^o of the saturated solution used. Afterwards, the sample thickness was measured to the nearest 0.001 mm using a digital micrometer (Mitutoyo, Kawasaki, Japan) at six different locations in each of the ten specimens.

Three batches of films (replicates) were prepared as indicated above. The film samples obtained from each batch were identified and distributed among the desiccators for storage at the RH (33.3%, 57.7%, or 75.2%) studied, in order to involve the influence of the film making in the following determinations.

Storage was performed under vacuum (P=132 Pa) with controlled RH in order to ensure that AA degradation initiated through the irreversible hydrolysis of its lactone ring as the first (and limiting) reaction step. Hence, the specific influence of water in the AA stability can be analyzed as a first approach for future investigations under normal air atmosphere and controlled RH of storage, in order to infer the specific influence of oxygen on the AA kinetics in darkness. The following analyses were performed on samples collected from the three batches at each time of interest.

Water Activity

The water activity was determined on the film samples with a Decagon AquaLab (Series 3 water activity meter, USA) at 25 °C (constant temperature) using a calibration curve made with the same standard saturated salt solutions mentioned above.

Determination of L-(+)-Ascorbic Acid

Film samples were taken from each of the three batches of films obtained in order to determine the AA kinetics from the triplicates of film making. Each film sample was first cut into pieces smaller than 1 mm in size, weighed on an analytical scale (0.0001 g), placed into a 25.00-ml volumetric flask with a 1% (*w*/*v*) oxalic acid solution, and submitted to magnetic stirring for 1.5 h at 5 °C to achieve the total extraction of AA from the film sample. During this time, it was also submitted to vortexing (Velp, Italy) for 90 s at 35 Hz, every 15 min. The suspension was finally centrifuged at 10,000 rpm and 6 °C for 30 min (Eppendorf 5810R Refrigerated Centrifuge, USA). An aliquot was taken from the supernatant, and the AA concentration was determined by using the 2,6-dichlorophenolindophenol spectrophotometric method (Rojas and Gerschenson 1991). The AA concentration was determined in two different aliquots (duplicate) for each film sample.

Measurement of pH

The measurement of pH was performed on the gel-forming solutions as well as on casted films after their equilibration at the corresponding RH. A bulb-combined glass electrode (Phoenix, AZ, USA) connected to a pH meter (Consort P901, ECC) was used for pH measurement of the gel-forming solutions. Alternatively, a flat surface combination electrode (Phoenix, AZ, USA) was used in the same arrangement to determine the film pH after a slight surface hydration with 20.0 μL of deionized water (Burin and Buera 2002). Calibrations were carried out with standard buffer solutions of pH 4.00 and 7.02.

Color

The measurement of the film color was accomplished with a Minolta colorimeter (Minolta CM-508d, Tokyo, Japan) using an aperture of 1.5 cm diameter. The exposed area was sufficiently large in relation to the illuminated area to avoid any edge effect. The instrument parameters used were a D-65 (sodium) illuminant and a 2° observer. The film samples were first rested on a white background standard. L, a, and b (HunterLab) values were averaged from five positions randomly selected across each 55-mm-diameter film sample (Trezza and Krochta 2000). Color parameters ranged from L=0 (black) to L=100 (white), $-a$ (greenness) to $+a$ (redness), and $-b$ (blueness) to $+b$ (yellowness). Standard values considered were those of the white background. White casting surfaces were used because the yellowness is determined as the deviation from whiteness according to the American Society of Testing and Materials (ASTM 1995). The yellowness index (YI) values of films were

calculated to report the NEB developed in film samples, according to the ASTM method D1925 (ASTM 1995). Film samples for color measurement were taken from each of the three batches of films obtained in order to determine the browning (YI) kinetics from the triplicates of film making.

Moisture or Water Content

For determination of the water content, film samples were taken 15 days after equilibration with the RH of storage. Each film was cut into pieces smaller than 1 mm in size, weighing (0.0001 g); placed into small, light glass containers; and dried in a vacuum oven at 70 °C until constant weight (around 30 days). Determinations were performed on six film specimens from each evaluated condition of RH, taking two film samples per batch.

Glass Transition Temperature

Differential scanning calorimetry (DSC 822^e Mettler Toledo calorimeter, Schwerzenbach, Switzerland) was used to determine the T_g (onset value; Miao and Roos 2004) from the second scan performed with the equilibrated film samples (10–15 mg) placed into a hermetically sealed 40-μL aluminum pressure pan. An empty pan served as reference. The first scan performed from −140 to 40 °C (10 °C/min) was followed by a second scan from −140 to 120 °C (10 °C/min), after cooling at 15 °C/min. An average value of replicates taken from the three batches of films and the corresponding standard deviation were reported. DSC was calibrated with the melting points of indium (156.6 °C), lead (327.5 °C), and zinc (419.6 °C), in addition to the periodic calibration performed with a sapphire disk, in the whole temperature range where the equipment is usually employed (Mettler 1997).

Proton Nuclear Magnetic Resonance Mobility

All the experiments were performed on the equilibrated film samples using a Bruker Avance II spectrometer operating at 300 MHz for ^{1}H. The probe was a Bruker high-power CP-MAS and was used under static conditions. The rotor sizes were 18 mm long with a 4-mm outer diameter. All the experiments were conducted on resonance at room temperature. The Carr Purcell Meiboom Gill pulse sequence was used to measure the transverse magnetization decay (T_2). The free induction decay was exported to WIN-NMR (Bruker) software where it was Fourier transformed and phase and baseline corrected. Overlapping spectra were deconvoluted by analysis. Peak intensity (area) and line-width of the deconvoluted peaks were recorded. The exponential curve fitting was performed at 50 and 10 ls using a nonlinear fitting program (OriginPro 7.5 SRO, Origin Lab Corporation, Northampton, MA, USA), and a Maxwell two-component curve was the best fitting model:

$$A_t = A_1 \exp\left(\frac{-t}{T_{2a}}\right) + A_2 \exp\left(\frac{-t}{T_{2b}}\right) \quad (1)$$

wherein T_{2a} and T_{2b} are the spin–spin relaxation times with "a" and "b" subscripts indicating the two components of the relaxation process, A_t = peak height, and A_1 and A_2= equilibrium magnetization. An average value of replicates taken from the three batches of films and the corresponding standard deviation were reported

Data Analyses

The results are reported as average and standard deviation. Rate constants of AA destruction were calculated by linear regression where AA concentration was in terms of grams AA/weight (grams) of the corresponding film sample assayed. The analysis of covariance was applied for the comparison of slopes, that is, of rate constants, as indicated by Sokal and Rohlf (2000). The statistical analyses of results were performed by applying analysis of variance (α, 0.05), followed by pair-wise multiple comparisons evaluated by Tukey's significant difference test (Sokal and Rohlf. 2000). The GraphPad Prism software (version 5.00, 2007, GraphPad Software Inc., USA) was used for all the analyses previously detailed.

Results and Discussion

Chemical Characterization of the Low Methoxyl Pectin Used

Pectin is a heterogeneous complex cell wall polysaccharide that is part of the human diet. Depending on its source and isolation procedure, pectin is polydisperse and polymolecular (Chandrasekaran 1998). The molecular weight (MW) of the LMP used for the film making was ≈734.2 kDa (mean value). It was similar to those of natural pectins extracted from cell walls of butternut pumpkin (Fissore et al. 2007) and sugar beet (Fishman et al. 2008).

The pectin backbone is made up of (1→4)-linked α-D-galacturonic acid (GalA) repeating units constituting HG segments or "smooth regions" of the macromolecule (Pérez et al. 2003; Vincken et al. 2003). Some of these carboxylic groups were methyl esterified as shown in Table 1, where the composition of the Genu™ LMP (with 32.0% *w/w* sucrose content) was summarized *after dialysis*. Commercial LMP is standardized with sucrose to a solid content between 30%

and 50% in order to modify the gelation temperature as well as specific gel properties like elasticity and syneresis. Pectin was 40% methyl esterified (DM, molar ratio) and also showed a DA=6% (Table 1). Substitution by acetyl groups can occur at C2 (2-O-acetyl-) or C3 (3-O-acetyl-) of the GalA monomers in HG segments (Ralet et al. 2008) and is expected to hinder an efficient packing of neighboring macromolecules and decrease the gelling ability, as typically observed in sugar beet pectins. The LMP herein used presented a GalA-to-Rha (molar) ratio of 13, as calculated from the composition shown in Table 1. It corresponds to ≈7.5% (molar ratio) of disorder (amorphous) regions of interspersed RG-I domains (Pérez et al. 2003). A galactose (Gal) to Rha (Gal/Rha) molar ratio of 1.2 and a Gal + arabinose to Rha [(Gal + Ara)/Rha] molar ratio of 1.35 were also calculated from data shown in Table 1. These ratios indicated that the chemical extraction of this commercial pectin produced hydrolysis or peeling of the hairy (RG-I) regions (Fry 1986; Fissore et al. 2010) of the original (cell wall) pectin macromolecules. Most of the pectin used by the food industry originates from citrus or apple peel from which it is extracted at a low pH and high temperature, being then primarily constituted by HG (Voragen et al. 2009).

Nowadays it is thought that low methyl-esterified pectins do not gel according to the egg box model in the presence of calcium ions (Braccini and Pérez 2001). Also, dimerization of antiparallel pectate chains by Ca^{2+} occurs through a cooperative binding even at very low polymer concentrations. Furthermore, the average demethylated block size of HG and the pattern of distribution of demethylated blocks produce pectins with different rheological properties and calcium reactivity, even though they have the same DM (Guillotin et al. 2005; Luzio and Cameron 2008). Among other ions like Mg^{2+}, Na^{+}, and K^{+}, the commercial LMP herein used carried 6.15×10^{-5} mol Ca^{2+}/g of pectin, as determined by atomic absorption spectrometry (Table 1). A calcium amount of 0.136 g was added per 5.50 g of LMP for gelling. It corresponded to ≈450 mol of Ca^{2+} per mole of pectin polymer (mean MW ≈734.2 kDa). By considering the pectin composition and molecular weights (Table 1), as well as the molar ratios between neutral sugars and GalA indicated above, it can then be calculated that ≈23% of the carboxylate groups of a pectin macromolecule can be electrostatically cross-linked by Ca^{2+} with other facing antiparallel chains.

In calcium pectate gels, the junction zones involve three components—uronate chains, calcium ions, and water—which correspond to non-freezable bound water (Ping et al. 2001). Water oxygen atoms may complete the coordination sphere of Ca^{2+} at the pectate junction zone, as observed by Braccini and Pérez (2001). Adequate distribution patterns of demethylated carboxyl groups (60% in the LMP herein used) are also necessary to constitute Ca junctions (Willats et al. 2001). These patterns are also associated with different degrees of water retention (Zsivanovits et al. 2004).

Previously, the AA stability was studied in a film network developed with a commercial HMP (GENU pectin type B, CP Kelco, NJ, USA) under a glycerol proportion of 36.8% *w*/*w* [glycerol.100/(pectin + glycerol)] (Pérez et al. 2009). In the present work, 46.8% *w*/*w* of glycerol was used in order to constitute LMP films with the same tensile resistance at failure: 660–1,700 N/m when equilibrated between 75.2% and 33.3% RH. The HMP showed an average MW of 457 kDa, being only constituted by 82% of GalA that was 73% methyl esterified and 14% acetylated (DA). A neutral sugar content of 18% was calculated, which was essentially Rha, Ara, and Gal. Molar ratios of GalA/Rha=30 and [(Ara+Gal)/Rha=6] were calculated, indicating a higher substitution at the RG-I regions than in the case of the LMP used in the present work.

Film Characteristics

The gelling film-forming solution contained AA, glycerol, and potassium sorbate as an antimicrobial agent, as well as the commercial (Genu™) or the pure (dialyzed) LMP (Table 1). The films made with dialyzed pectin permitted to determine the influence of sucrose on the AA degradation and browning development.

Homogeneous and flexible films were obtained by casting from both LMP herein used, being plasticized with 46.8% *w*/*w* of glycerol. The films were transparent and slightly yellow (b=+10.0, YI=22±1), showing high lightness (L=81.8±0.5%). The initial AA concentration experimentally determined on samples of the casted films was ≈3.00% (*w*/*w*), calculated on the basis of the film. This value meant that the AA retention reached after drying was 100%.

Kinetics of L-(+)-Ascorbic Acid Degradation and Non-Enzymic Browning Development

The film samples stored at a constant temperature (25 °C) and RH (33.3%, 57.7%, or 75.2%) reached the equilibrium on the fourth day of storage as determined from the measurement of the film a_W (0.333, 0.577, or 0.752) every day. After equilibration, the film thickness was 0.104± 0.004 mm, and the pH changed into a value of 3.38±0.03 during film storage.

The ratio between the AA concentration [$C_{AA}(t)$] and the initial one [C_{AAO}] changed with storage time according to a pseudo-first-order (p<0.05) kinetic (Fig. 1a). On the other hand, the browning development measured as the increment

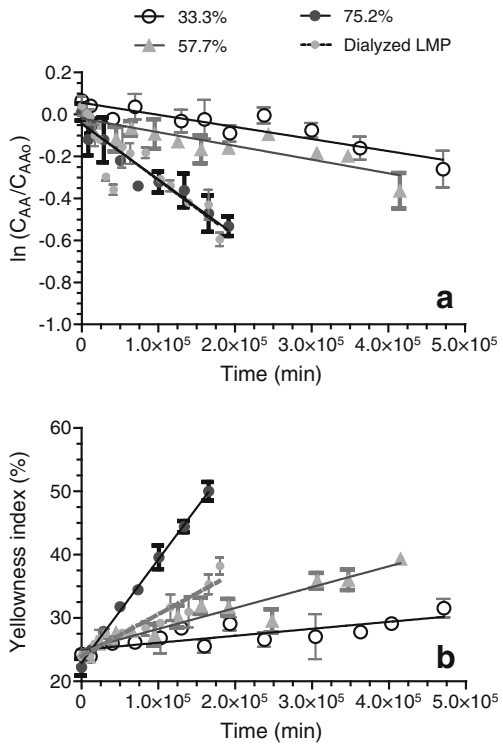

Fig. 1 Kinetics of L-(+)-ascorbic acid (AA) loss (**a**) and NEB (YI, yellowness index) development (**b**) into LMP films stored at constant relative humidity and 25 °C. Kinetics of films made with dialyzed LMP is also shown for the system equilibrated at 75.2% RH. *Error bars* indicate the standard deviations (n=3)

of the yellowness index (YI) with the time of storage statistically fitted (p<0.05) to a pseudo-zero-order reaction (Fig. 1b), as often reported (Saguy et al. 1978; Labuza and Baisier 1992; Rojas and Gerschenson 2001; Torregrosa et al. 2006; Patras et al. 2009). As observed in Fig. 1, equilibration time of the casted films at each RH did not seem to affect the kinetics of AA destruction or browning increment from zero time (t=0 min). There were no significant differences between the AA loss rates on the films made directly with commercial LMP (CP Kelco) and with dialyzed LMP. This can be observed in Fig. 1a where the kinetics of AA degradation in dialyzed LMP films equilibrated at 75.2% RH was also included as an example.

The rate constants of AA destruction (k_{AA}') and NEB development (k_{YI}) calculated from data regression are summarized in Table 2. They increased with the RH of storage, where the browning (film color development or YI increase) was more sensitive to the a_W (RH%/100) of the *solid-like* system than the AA degradation (Fig. 2a). In reference to the degree of methylesterification of the pectin used, k_{AA}' and k_{YI} kinetic parameters were lower than those obtained from films previously developed with HMP (DM of 73%) by Pérez et al. (2009) (Fig. 2b). The AA supported by the films made with commercial LMP degraded slowly as indicated by the half-life time ($t_{1/2}$) calculated from the kinetic rate constants (k_{AA}') determined after the film storage (Table 2).

Table 2 Kinetic parameters for L-(+)-ascorbic acid (AA) destruction and browning development, as well as physical and chemical characteristics of the low methoxyl pectin films determined after storage at constant relative humidity (25 °C)

	Relative humidity		
	33.3%	57.7%	75.2%
k'_{AA}[a] (min^{-1})	(6.5±0.9)×10^{-7} A	(6.6±0.8)×10^{-7} A	(26±2)×10^{-7} B
k_{YI}[a,b] (YI units min^{-1})	(1.1±0.2)×10^{-5} A	(3.3±0.2)×10^{-5} B	(16,4±0.3)×10^{-5} C
Lightness[c], L (%)	82±1 A	81±1 A	81±1 A
Half-life time of L-(+)-ascorbic acid ($t_{1/2}$, days)	744 A	727 A	185 C
Moisture content[d] (g water/100 gdm)[e]	12,5±0.5 A	23.6±0.4 B	33±2 C
Glass transition temperature (onset), T_g (C)	−79.45 A	−93.99 B	−103.93 C
Change in specific heat at the glass transition [J/g (dm) K][e]	0.807 A	1.065 B	1.458 B
k_{YI}[a,b] (YI units min^{-1}) (dialyzed pectin films)	(1.3±0.4)×10^{-5} A	(3.0±0.2)×10^{-5} B	(6.5±0.4)×10^{-5} C
Glass transition temperature (onset), T_g (°C) (dialyzed pectin films)	−80.13 A	−95.22 B	−102.41 C

The same capital letter in each row indicates non-significant differences (p<0.05) between values

[a] The kinetic rate constant and standard deviation are shown (n=3). They were obtained from measurements performed on three batches of films or three experiments

[b] YI, yellowness index for reporting non-enzymic browning development

[c] The arithmetic mean and standard deviations are shown (n>11)

[d] The arithmetic mean and standard deviations are shown (n=6)

[e] dm, dry matter

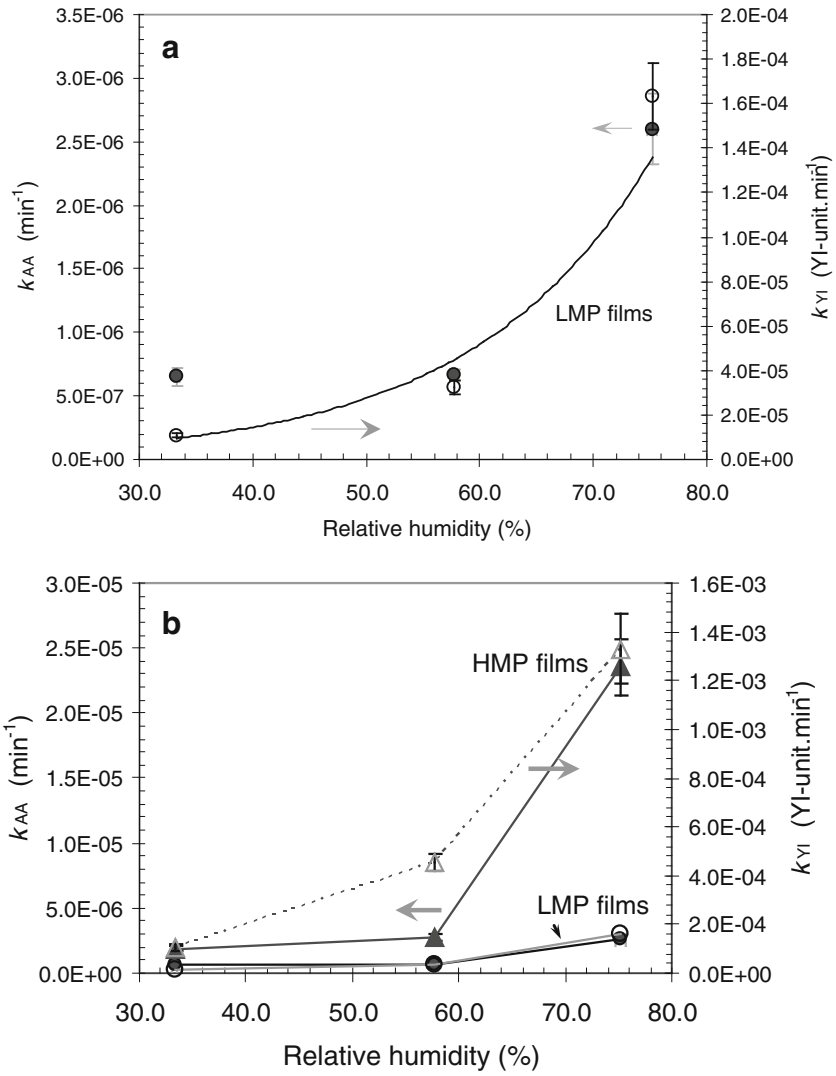

Fig. 2 Rate constants for AA loss (k'_{AA}; *filled symbols*) and NEB development (k_{YI}; *empty symbols*) determined in LMP (*white circles*) (**a**, **b**) as well as in HMP (*white triangles*) (**b**) (Pérez et al. 2009) films, are plotted against relative humidity of film storage at 25 °C. *Error bars* indicate the standard deviations (n=3)

Moisture Content, Water Activity, and Macromolecular Relaxation

A substantial decrease ($\Delta T \approx 24$ °C) in the T_g was observed with the increment of the RH in storage and also of the moisture content (Table 2). This meant that water was being compromised in plasticization leading to macromolecular relaxation. The T_g values previously determined on film networks based on HMP are also plotted in Fig. 3. Very similar macromolecular mobility was observed since LMP and HMP film systems were almost in the same T_g range. They were amorphous-rubber systems at 25 °C of storage. This high macromolecular mobility came essentially from polymer plasticization by glycerol, whose earlier presence in the films allowed the subsequent water penetration in the network during equilibration (Pérez et al. 2009). Besides, the DSC scans obtained from LMP films (46.8% *w/w* of glycerol), equilibrated either at 33.3%, 57.7%, or 75.2% RH, did not show any other phenomenon beyond the T_g when sweeps were performed at 10 °C/min between −140 and +110 °C. By contrast, the films developed with HMP (36.84% *w/w* of glycerol ratio) and equilibrated at 75.2% RH additionally showed an endothermic peak at ≈−34 °C (Pérez et al. 2009) attributable to freezable bound water (Hatakeyama and Hatakeyama 1998). Ping et al. (2001) indicated that beyond a certain water content threshold, the water molecules sorbed to hydrophilic polymers become freezable, but with a melting point lower than 0 °C due to their location in the second hydration level. Hence, water seemed to be certainly less available in the LMP network herein formulated, even at the highest RH in storage (75.2%) and with similar moisture contents.

Moisture Content, Water Activity, and Chemical Reactions Involved

Differences in AA stability and browning rates as a result of RH of film storage (Table 2, Fig. 2) indicate the influence of

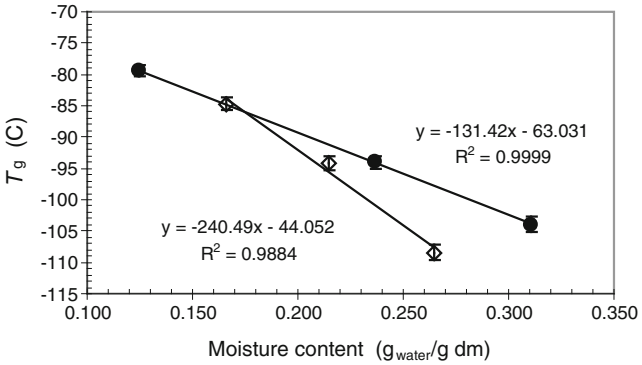

Fig. 3 Glass transition temperature (T_g) against moisture content plotted for LMP (*black circles*) as well as for HMP (*white diamonds*) (Pérez et al. 2009) films stored at constant relative humidity and 25 °C. *Error bars* indicate the standard deviations (n=6); *dm* dry matter

the water availability in order to accomplish the chemical reactions involved. In the present work, storage was performed under vacuum (P=132 Pa). This is necessary for ensuring that AA degradation initiated through the irreversible hydrolysis of its lactone ring as the first reaction step (Kurata and Sakurai 1967). León and Rojas (2007) proposed that a water molecule may be involved in a bimolecular nucleophilic substitution mechanism (S_N2) at the first step of the chemical destruction of AA under anaerobic storage. This reaction leads to the hydrolysis of the AA-lactone ring to irreversibly render 2-keto-L-gulonic acid (KGA) through an acid-catalyzed reaction (Kurata and Sakurai 1967). The attack of the AA (lactone) ring by a water molecule (S_N2) was also suggested to be slow enough in order to determine the total reaction rate. This proposed mechanism explains a second-order kinetics for AA hydrolysis (Morrison and Boyd 1990),

$$r_{AA} = -\frac{1}{\nu_{AA}}\frac{d[AA]}{dt} = k[H_2O][AA] = k'_{AA}[AA] \qquad (2)$$

wherein ν_{AA} is the stoichiometric coefficient for AA hydrolytic degradation reaction (here ν_{AA}=1), r_{AA} is the AA-reaction rate/unit volume at a constant temperature, [AA] is the molar concentration of AA, [H_2O] is the molar concentration of water, k is the rate constant of the second-order kinetics for AA hydrolytic degradation reaction, and k'_{AA} is the rate constant of the pseudo-first-order kinetics for AA hydrolytic degradation reaction. Hence, the water concentration takes part in the rate constant (k'_{AA}) since it is determined from the measurement of the AA concentration remaining after every trial (Fig. 1a). Consequently, water availability is a feature in the pseudo-first-order rate constant (k'_{AA}) obtained by statistical fitting of the AA data (Fig. 1a). Since the availability of the adsorbed water increased as the *solid-like* (film) networks were stored between 33.3% and 57.7% and 75.2% RH, k'_{AA} also increased for AA destruction with the same trend. Water adsorbed in solid-like or polymeric systems equilibrated at the water activities herein studied (0.333–0.752) is a limiting reactive. Water is not available as solvent in film systems. Conversely, water is the solvent in aqueous solutions. It produces solvation of anions and other reactive compounds and, hence, an important decrease in their reactivity.

Once KGA is produced after the hydrolysis of the AA-lactone ring, this reactive molecule (with α,β-unsaturated carbonyl and β-hydroxy carboxyl groups) goes through successive transformations that involve dehydrations and decarboxylations, producing different browning active compounds (Kurata and Sakurai 1967). The absence of water available for solvation in the film (solid like) networks (33.3–75.2% RH range) promotes dehydration reactions as well as high reactivity of nucleophiles (Morrison and Boyd 1990). This allows concluding that the hydrolysis of AA to produce KGA may be in fact a decisive path that may control the NEB in solid-like systems like LMP films. Browning (a color appearance) seemed to be more dependent on water *availability* than k_{AA}', at 33.3% and 57.7% RH (Fig. 2a). Probably, this may in part be due to the fact that pectin macromolecules, as other biopolymers, might provide some activity as an acidic catalyst on browning reactions, an activity coming from the presence of carboxylic and hydroxyl groups. It is suggested that the polysaccharide network may be in part activated like catalyst as water is highly constrained in the film systems (Corain et al. 2001), suggesting that polymers are not necessarily inert in the presence of water and small entrapped molecules.

In general, browning parallels AA destruction (Fig. 1). There were no significant differences between the rate constants of YI increment in the films made directly with commercial (CP Kelco) and dialyzed pectin after equilibration at 33.3% or 57.7% RH. Lower browning rate was only observed at 75.2% RH in the films developed with dialyzed LMP (Fig. 1b).

Water Dynamic at Molecular Level: Influence of Sucrose Presence and Degree of Methylation

Since the rate constants (k_{AA}', k_{YI}) were dependent on the RH of the film storage, the dynamic aspects of water interactions in the complete formulation of edible film networks were studied. For low-moisture biopolymer systems (water content <35%), the slowing of water motion has been reported to be associated with bound water (i.e., immobile water) arising from hydrogen bonding (Kou et al. 2000). Short-range motions or molecular (water) relaxations investigated in the present work by ^{1}H-NMR (Vittadini and Chinachoti 2003) led to observe that the magnetization decay in the x–y plane showed two spin–spin time constants (T_{2a} and T_{2b}) as observed after exponential

fitting (Eq. 1), indicating the existence of multi-relaxation rate behavior. These parameters may be associated with two fractions of water, which have different relaxation rates or mobility degrees (Chen et al. 1997; Kerr and Wicker 2000). One of the fractions showed transverse relaxation values between 1.68×10^{-4} and 1.058×10^{-3} s (T_{2a}), while the other water population presented characteristic times of transverse relaxation ranging from 1.13×10^{-3} to 4.33×10^{-3} s (T_{2b}). Non-significant different values were shown by the films made with dialyzed LMP except for the samples equilibrated at 75.2% RH, whose T_{2a} and T_{2b} were 7.80×10^{-4} (Fig. 4) and 3.41×10^{-3} s, respectively. This may indicate some more restrictive effects of the plasticized polymers on the surrounding water molecules in the absence of sucrose. The lower water mobility determined on dialyzed LMP films equilibrated at 75.2% RH may be in part coherent with its lower browning rate (Fig. 4), but it did not significantly affect the AA loss. Hence, sucrose seemed to hinder the water retention caused by the pectin network, increasing the water mobility in the glycerol-plasticized network and also the browning development from it. Rate constants of AA loss (k'_{AA}) in the film networks developed either from commercial or from dialyzed LMP did not change when equilibrated at 75.2% RH. Firstly, sucrose has to hydrolyze in D-glucose and D-fructose that participate in browning reactions. This hydrolysis can be favored by some "acidic" medium created in the film network by the LMP and AA. Thus, it is the water dynamic that seems to in part justify the AA stabilization achieved in the LMP networks equilibrated at 33.3%, 57.7%, or 75.2% RH and probably not the macromolecular mobility per se. Though with a lower glycerol proportion than the LMP films herein studied, the HMP film developed by Pérez et al. (2009) showed a higher ($p<0.05$) value of T_{2b} for films stored at 75.2% RH. Transverse relaxation values were between 1.986×10^{-4} and 1.038×10^{-3} s for T_{2a} and from 1.184×10^{-3} to 7.446×10^{-3} s for T_{2b} in films made with HMP. Both T_{2a} and T_{2b} were significantly ($p<0.05$) higher than the ones determined in the present work for films made with dialyzed pectin and equilibrated at 75.2% RH. All these results seemed in part to coincide with the fact that LMP films did not show any evidence of endothermic peaks corresponding to freezable bound water at −34 °C from DSC scanning. This is in contrast to that previously observed in the HMP films when stored at 75.2% RH (Pérez et al. 2009). A higher proportion of methyl-esterifying groups in the pectin macromolecules of HMP films led to a significant level of hydrophobic interactions from hydration of apolar (methyl) groups. This may enhance water–water (or, in the present case, water–glycerol) hydrogen bonding over the weaker methyl group–water interactions (Mathlouthi 2001), contributing to lower water immobilization by the HMP macromolecules.

From all previously considered, it can be suggested that some higher restriction of water mobility in the LMP network can in part arise from water interaction with the polymer. This may concern water interacting with the coordination sphere of Ca^{2+} from the moment at which the film network developed during gelling and casting (Ping et al. 2001; Braccini and Pérez 2001). After this, equilibration at different RH affected macromolecule relaxation as determined from the T_g values. However, water was more confined in the LMP networks as indicated by the order of magnitude of their T_{2a} and T_{2b} values in dialyzed LMP films. Ping et al. (2001) reported that the average number of non-freezable water molecules per site depends on the chemical nature of the polar site. For a polymer with carboxylate sites, it increases with the size of the alkaline counterion (herein Ca^{2+}) of the site due to the increasing ability of the [carboxylate–counterion] pair to undergo dissociation. Carboxylate groups are involved in the Ca^{2+} coordination at the

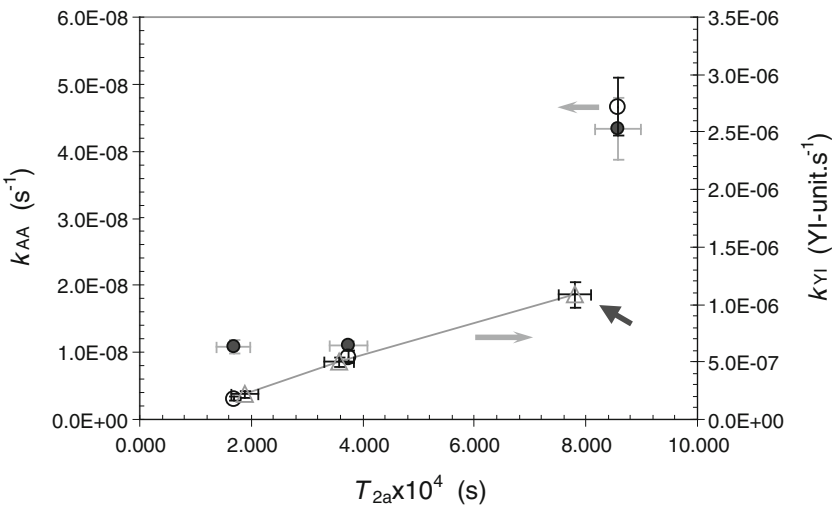

Fig. 4 Rate constants for AA loss (k'_{AA}, *black circles*) and NEB development (k_{YI}, *white circles*) plotted against the spin–spin relaxation time (T_{2a}) for LMP films (25 °C). The k_{YI} of films made with dialyzed LMP and equilibrated at 75.2% RH (*arrow*) was only different (*white triangle*). *Error bars* indicate the standard deviation for k ($n=3$) and T_{2a} ($n=9$)

junction zones. Otherwise, the glycosidic linkages and –OH groups can interact with glycerol as well as with water adsorbed (Pérez et al. 2009). Sucrose present in the commercial LMP (CP Kelco) may also contribute to the water constraint (Mathlouthi 2001) but the glycerol-plasticized polymer can mainly exert the decisive retention of water molecules in the casted (solid-like) materials.

About 23% of the GalA residues of each LMP macromolecule (MW≈730.2 kDa) would be related through calcium junction zones as can be calculated from the pectin composition (Table 1) and moles of Ca^{2+} added. At the same time, the distribution pattern of demethylated (60%) blocks in the HG smooth regions of the LMP used may also be critical (Willats et al. 2001) since these patterns are also associated with different degrees of water retention (Zsivanovits et al. 2004).

Conclusions

The LMP used in the present work, either applied directly in its commercial form or after dialysis in order to eliminate sucrose, constituted edible films that were able to significantly stabilize AA in the presence of water adsorbed after equilibration at 33.3% ($t_{1/2}$=744 days), 57.7% ($t_{1/2}$= 727 days), or 75.2% ($t_{1/2}$=185 days) RH, with the subsequent inhibition of browning. Some higher restriction of water mobility in the LMP network than in the HMP films can arise more probably from the water interaction with the macromolecules. Equilibration at the studied range of RH affected macromolecular relaxation, and it was done in a similar degree (T_g≈−90 °C) to the one observed in the case of HMP films. These T_g values were associated to the network plasticization by glycerol. Beyond this, water seemed to be more confined in the LMP networks as indicated by the order of magnitude of T_{2b} as well as by the absence of non-freezable bound water peak observed at 75.2% RH from DSC. The restriction of water mobility by the LMP polymer can mainly be associated to water interacting with the coordination sphere of Ca^{2+}. Sucrose present in the commercial LMP (CP Kelco) may also associate water, but the polymer seemed to exert a decisive effect concerning the retention of water molecules in the casted materials as observed at 75.2% RH. Conversely, hydrophobic interactions from hydration of apolar methyl groups weakened water interaction with the polymers in the case of the HMP films, contributing to lower water immobilization and, hence, to significantly higher AA loss and browning rates.

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