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Storage stability of laccase induced arabinoxylan gels

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Abstract

The effect of storage on laccase-induced gels of wheat water-extractable arabinoxylan (WEAX) was followed for 6 days at 25 °C. Gel hardness was greatly affected by aging (43% decrease in 6 days). This weakening appeared to proceed through a mechanism involving laccase-produced radicals. Once produced, they participated in secondary reactions leading to a slight degradation of WEAX main chains (20% decrease of M_W and η_{red} values) and a decrease by 70 and 80% of their di and tri-ferulic acid content, respectively. The thermal inactivation of laccase after gel formation blocked the free radical production thereby stabilizing the gel. The changes in hardness (5% lost), M_W (5% lost) and η_{red} (1% lost) were reduced in this case and the contents of di and trimers of ferulic acid were not modified.

Keywords: Arabinoxylan gel; Ferulic acid; Diferulic acid; Triferulic acid; Aging; Free radicals

1. Introduction

Arabinoxylans are important cereal non-starch polysaccharides constituted of a linear backbone of β-(1→4)-linked D-xylopyranosyl units to which α-L-arabinofuranosyl substituents are attached through O-2 and/or O-3 (Izydorczyk & Biliaderis, 1995). Some of the arabinose residues are ester-linked on (O)-5 to ferulic acid (FA) (3-methoxy, 4 hydroxy cinnamic acid) (Smith & Hartley, 1983). These polysaccharides have been classified as water extractable (WEAX) or water-unextractable (WUAX). WEAX form highly viscous solutions and can gel through ferulic acid covalent cross-linking upon oxidation by some chemical or enzymatic free radicals-generating agents (Figueroa-Espinoza & Rouau, 1998; Geissman & Neukom, 1973; Hoseney & Faubion, 1981; Izydorczyk, Biliaderis, & Bushuk, 1990). Laccase (*p*-diphenol oxygen oxidoreductase, EC 1.10.3.2), blue multi-copper enzyme of white rot fungi (Bollag & Leonowicz, 1984; Holwerda, Wherland, & Gray, 1976) can oxidize FA beared on WEAX resulting in the formation of five diferulic acids (di-FA) (5-5′, 8-5′ benzo, 8-*O*-4′, 8-5′ and 8-8′ di-FA), the 8-5′ and the 8-*O*-4′ forms being always preponderant (Figueroa-Espinoza, Morel, & Rouau, 1998; Vansteenkiste, Babot, Rouau, & Micard, 2004). These di-FA structures were first identified in grass cell walls by Ralph, Quideau, Grabber, and Hatfield (1994). Di-FA covalent cross-linking have been commonly considered as firstly responsible of the WEAX gel development (Figueroa-Espinoza & Rouau, 1998; Geissmann & Neukom, 1973; Izydorczyck & Biliaderis, 1995) The implication of higher coupling products of FA in WEAX cross-linking has been suggested by Vansteenkiste et al. (2004) studying the cross-linking of wheat WEAX by laccase. However, although two trimers of FA (from the addition of a phenoxy radical to the decarboxylated 8-5′or 8-*O*-4′-di-FA) have been reported in FA polymerized by a lignin peroxidase (Ward, Hadar, Bilkis, Konstantinovsky, & Dosoretz, 2001) and the 4-*O*-8′, 5′-5″-dehydrotriferulic acid has been identified in maize bran (Bunzel, Ralph, Funk, & Steinhart,

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2003; Rouau et al., 2003), evidence of such cross-links products in enzymatically induced WEAX gels has not yet been reported.

WEAX gels possess interesting functional properties for food applications thanks to their neutral taste and odour, high water absorption capacity (up to 100 g of water per gram of dry polymer) and absence of pH or electrolyte susceptibility (Izydorczyk & Biliaderis, 1995). Unlike most of the polysaccharide gels currently used as texturizing and stabilizing agents in food systems, WEAX gels are mostly stabilized by covalent linkages. However, both covalent and non-covalent linkages are thought to participate in the gel structure (Izydorczyk & Biliaderis, 1995; Rattan, Izydorczyk, & Biliaderis, 1994; Vansteenkiste et al., 2004). Covalently cross-linked gels are generally strong, form quickly, are stable upon heating and exhibit no syneresis after long time storage.

The stability of hydrogels used in food applications is a major problem, as final products must keep their properties throughout their shelf life. Up to now the literature devoted to WEAX gels (Dervilly-Pinel, Rimsten, Saulnier, Andersson, & Åman, 2001; Figueroa-Espinoza & Rouau, 1999; Izydorzyck et al., 1990; Vansteenkiste et al., 2004; Vinkx, Van Nieuwenhove, & Delcour, 1991) only reported on cross-linking kinetics of WEAX or on properties of freshly cured gels. In this work, we have followed the evolution of rheological, chemical and physicochemical properties of laccase-induced WEAX gels during a 6-day period.

2. Experimental

2.1. Materials

Wheat water extractable arabinoxylans (WEAX) were obtained and characterized as reported by Vansteenkiste et al. (2004). Laccase (benzenediol:oxygen oxidoreductase, E.C.1.10.3.2) from *Pycnoporus cinnabarinus* was supplied by the Unité de Biotechnologie des Champignons Filamenteux-(UMR 1163 INRA-ESIL, Luminy, France). Citric acid, sodium phosphate dibasic, syringaldazine, ferulic acid and TMCA (3,4,5-trimethoxy-*trans*-cinnamic acid) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Lithium nitrate was purchased from Fluka Chemical Co. (Buchs, Switzerland).

2.2. Methods

2.2.1. Preparation of WEAX gel

One percent and 2% (w/v) pure WEAX solutions were prepared in 0.05 M citrate phosphate buffer pH 5.5. The buffer was filtered through 0.2 μm (Whatman) to prevent microbial contamination. Laccase (1.675 nkat/mg WEAX) was used as cross-linking agent. Gels were allowed to form for 2 h at 25 °C. For the chemical and physicochemical analysis, gels (1 ml of 1% (w/v) WEAX) were formed in 10 cm hermetically closed glass-tubes. Concerning the rheological properties, small deformation tests were performed onto 1% WEAX gels (2 ml) directly made on the rheometer. Large deformation tests necessitate the use of 2% (w/v) WEAX gels (6 ml in glass flasks of 30 mm height and 25 mm internal diameter) in regard to the sensibility limit of the method.

2.2.2. Laccase activity

Laccase activity was measured at 25 °C using a laccase solution at 0.125 mg/ml dissolved in 0.1 M citrate–phosphate buffer pH 5.5 (Figueroa-Espinoza & Rouau, 1998). Syringaldazine 0.0216 mM in methanol was used as substrate. The enzymatic reaction was followed during 4 min at 530 nm. Laccase activity was 3910.5 nkat/mg. Under the WEAX gelation conditions used in this study, laccase presented no detectable xylanase activity on a cross-linked wheat arabinoxylan substrat (Megazyme endo-1,4-β-xylanase assay) even after 6 days at 25 °C.

2.2.3. Laccase thermal inactivation

Laccase was inactivated by a 90 °C–15 min treatment of the freshly cured gel (gels were allowed to form for 2 h at 25 °C). Treatment was realised in a water bath (Lauda E100, Brinkmann Instruments, Westbury, USA). The efficiency of the laccase inactivation treatment was preliminary checked by measuring the residual enzymatic activity as described above after different times of thermal incubation. A 100% laccase inactivation was obtained after 15 min exposure at 90 °C.

2.2.4. Rheology

2.2.4.1. Small deformation measurements. The formation of the WEAX gel was followed using a strain-controlled rheometer (ARES 2000, Rheometric Expansion System, Rheometric Scientific, Champ sur Marne, France) in oscillatory mode as follows. Cold (4 °C) solutions of 1% (w/v) WEAX were mixed with laccase (1.675 nkat/mg WEAX) and immediately placed in the cone and plate geometry (5.0 cm in diameter, 0.04 rad in cone angle) maintained at 4 °C. Exposed edges of the sample were covered with silicone fluid to prevent evaporation during measurements. WEAX gelation kinetic was started by a sudden increase in temperature from 4 to 25 °C and monitored at 25 °C for 2 h by following the storage (G') and loss (G'') modulus. All measurements were carried out at a frequency of 1.0 Hz and 10% strain (linearity range of visco-elastic behavior). Frequency sweep (0.16–16 Hz) was carried out at the end of the network formation at 10% strain and 25 °C.

2.2.4.2. Large deformation measurements. The strength of control (25 °C) and heat treated (90 °C–15 min) 2% (w/v) WEAX gels, freshly made (2 h) and aged (6 days–25 °C), was analyzed with a TA.XT2 Texture Analyzer (RHEO

Stable Micro Systems, Haslemere, England) equipped with a XTRAD software version 3.7. The gels were deformed by compression at a constant speed of 1.0 mm/s to a distance of 4 mm from the gel surface using a cylindrical plunger (diameter 25.4 mm). The peak height at 4 mm compression was called gel hardness (Czuchajowska, Otto, Paszczynska, Bozena, & Byung-Kee, 1998; Mao, Tang, & Swanson, 2001; Yamin, Lee, Pollak, & White, 1999).

2.2.5. Chemical and physicochemical analyses

2.2.5.1. Ferulic acid (FA), dimers of ferulic acid (di-FA) and trimer of ferulic acid (tri-FA). Gel FA and di-FA contents were quantified by high performance liquid chromatography (HPLC) after deesterification step as described by Vansteenksite et al. (2004). Tri-FA (4-*O*-8′, 5′-5″-dehydro-triferulic acid) levels were quantified as described by Rouau et al. (2003). FA, di-FA and tri-FA levels were followed during gel formation at 0, 10, 20, 30, 40 and 120 min and during gel aging at 0, 4, 10 and 14 h and at 1, 2, 4 and 6 days at 25 °C. An Alltima C_{18} column (250×4.6 mm) (Alltech associates, Inc., Deerfield, IL) and a photodiode array detector Waters 996 (Millipore Co., Milford, MA) were used. Detection was by UV absorbance at 320 nm.

2.2.5.2. WEAX molecular weight. One milliliter of 1% (w/v) WEAX gels was disrupted by release of phenolic bridges with 0.5 ml 0.5 N NaOH during 15 min at 25 °C. The pH was then immediately adjusted to 7 with 2 N HCl to prevent WEAX chain degradation. Finally, the samples were filtered through 2.7 μm (Whatman) and analyzed by size exclusion high performance liquid chromatography (SE-HPLC) at 38 °C using a Ultrahydrogel 1000 Waters (Millipore Co., Milford, MA) column (7.8×300 mm), with pullulan limit exclusion of 10^6 Da. Column was eluted isocratically by 0.2 M $LiNO_3$ (filtered through 0.2 μm) at 0.6 ml/min. 20 μl of sample were injected and a Waters 600 differential refractometer was used for detection.

2.2.5.3. Viscosity determinations. Specific viscosity (η_{sp} of WEAX solutions or NaOH disrupted WEAX gels was measured with an AVS 400 capillary viscosimeter (Schott Geräte, Hofheim, Germany), equipped with an Oswald capillary tube (flow water time 75.15 s). The η_{sp} was related to the WEAX concentration (η_{sp}/C) to obtain their reduced viscosity η_{red} (ml/g) according to Rao (1993). The intrinsic viscosity [η] was determined by the Mead, Kraemer and Fouss method (Kraemer, 1938; Mead & Fouss, 1942).

2.2.6. EPR analysis

The production of free radicals during laccase action on 1% (w/v) WEAX solutions was followed by using electron paramagnetic resonance (EPR) spectroscopy. X-band (9.7 GHz) EPR spectra were recorded at room temperature using an ESP 300E Bruker spectrometer equipped with a high sensitivity cylindrical cavity. A capillary tube of 1 mm inner diameter (30 μl) was filled with a mixture of 1% (w/v) WEAX solution and laccase (1.675 nkat/mg AX). The spectra were recorded with the following parameters: microwave power 10 mW, modulation amplitude 0.8 mT, modulation frequency 100 kHz, sweep width 10 mT centered at $B=347$ mT and acquisition time 40 s for one scan. For spin quantitation, the second integral value of the radical spectrum was compared with that given by a $CuSO_4$ standard recorded at room temperature under non-saturating conditions.

2.2.7. Statistical analysis

Small deformation rheological measurements of gelation were made in triplicate. Large deformation rheological measurements, chemical and physicochemical analyses were made in duplicate. The coefficients of variation for the measured values for phenolic acids, molecular weight and viscosity determinations were lower than 5%. Hardness, molecular weight and reduced viscosity data were subjected to variance analysis (ANOVA) with treatment (heat) and storage time as factors of variation. These data were compared by Duncan's multiple-range test. A 95% confidence level was used. Results are expressed as mean values.

3. Results and discussion

3.1. Characterization of WEAX

WEAX were obtained from a mixture of Soissons, Thésée and Apollo wheat cultivars as described by Vansteenkiste et al. (2004). Their composition is presented in Table 1. Galactose residues were detected due to the coextraction of arabinogalactan-proteins (Fincher & Stone, 1974). Nevertheless, after correction of arabinose from arabinogalactan-proteins (Ara/Gal=0.7; Loosveld, Maes, Van Casteren, Schols, Grobet, & Delcour, 1998), arabinoxylan was the main polysaccharide in the sample (62% db). WEAX presented a 0.6 arabinose-to-xylose ratio, which agrees with other wheat endosperm WEAX (Figueroa-Espinoza & Rouau, 1998; Izydorczyk, Biliaderis, & Bushuk, 1991a). Its molecular weight (M_w) and intrinsic

Table 1
Composition of wheat WEAX

Component	Dry matter (mg/g)
Arabinose	247.5±7.9
Xylose	371.4±25.4
Glucose	21.8±1.4
Galactose	69.4±3.2
Protein	40.6±0.3
Ferulic acid	1.70±0.01
Diferulic acids	0.150±0.001
Triferulic acid	0.010±0.001

All values are average from two repetitions.

viscosity [η] values were 480 kDa and 469 ml/g, respectively. WEAX contained 1.70 ± 0.01 μg FA/mg and 0.150 ± 0.001 μg total di-FA/mg, values close to that reported in other wheat flour WEAX (Figueroa-Espinoza et al., 1998; Schooneveld-Bergmans, Dignum, Grabber, Beldman, & Voragen, 1999). The relative percentages of each di-FA present in the WEAX were approximately 51, 33 and 15 for the 8-5′(mainly under benzofuran form), 8-*O*-4′ and 5-5′ structures, respectively. The 8-8′ di-FA was not detected in our study. The predominance of 8-5′ and 8-*O*-4′ dimer structures had also been recently reported by Dervilly-Pinel et al. (2001) and Vansteenkiste et al. (2004) in arabinoxylans from wheat flour. A trimer of ferulic acid (4-*O*-8′, 5′-5″) was detected in very low amounts (0.01 ± 0.001 μg tri-FA/mg WEAX).

3.2. WEAX gel formation

The formation of the WEAX gel was rheologically investigated by small amplitude oscillatory shear. Fig. 1a shows the development of G' and G'' moduli of a 1% (w/v) WEAX solution undergoing gelation by the laccase. The gelation profile followed a characteristic kinetics with an initial increase of G' followed by a plateau region. The values of G' and G'' at the plateau region (120 min) were 14.6 and 1.4 Pa, respectively. Izydorczyk, Biliaderis, and Bushuk (1991b) reported G' values from 3.2 to 36.7 Pa for 2% (w/v) arabinoxylans gelled by peroxidase/H_2O_2 system from different wheat varieties. The mechanical spectrum of the gel obtained for 2 h gelation (Fig. 1b), was typical of a solid-like material with a linear elastic modulus (G') independent of frequency and G'' much smaller than G' and dependent of frequency (Clark, 1992). This behavior is similar to that reported for arabinoxylans from rye, triticale (Dervilly-Pinel et al., 2001) and wheat (Izydorczyk et al., 1990; Vansteenkiste et al., 2004) gelified by a laccase or peroxidase/H_2O_2 system. The gelation profile was similar to that reported elsewhere for arabinoxylans from wheat and barley endosperm treated with a peroxidase/H_2O_2 system (Dervilly-Pinel et al., 2001; Izydorczyk & Biliaderis, 1995; Rattan et al., 1994). Ferulate monomers, dimers and trimers were measured during the gelation process (at 0, 10, 20, 30, 40 and 120 min) (Fig. 1a). Typical and simultaneous FA oxidation and di-FA formation were observed as previously reported by Figueroa-Espinoza & Rouau (1998) and Vansteenkiste et al. (2004) on wheat WEAX treated with laccase. FA was mainly oxidized (73% of initial FA content) during the first 30 min of the reaction leading to the accumulation of the maximal amount of dimers (0.55 ± 0.07 μg/mg WEAX) and tri-FA (0.074 ± 0.001 μg/mg WEAX) when G' reached 80% of its final value (Fig. 1a). From 30 to 120 min, the tri-FA levels remained constant, while the di-FA levels slightly decreased from 0.55 ± 0.07 to 0.49 ± 0.02 μg/mg WEAX. These results indicate that the formation of di-FA and tri-FA occurred simultaneously during the first 30 min of gelation and that the diminution of di-FA + FA levels after 30 min of gelation did not generate the tri-FA formation. A similar behavior of G' evolution with FA and di-FA changes during wheat WEAX laccase gelation was recently reported by Vansteenkiste et al. (2004). These authors suggested the involvement of non-covalent interactions between adjacent WEAX chains or the formation of WEAX cross-links by oligomers of ferulic acid higher than dimers.

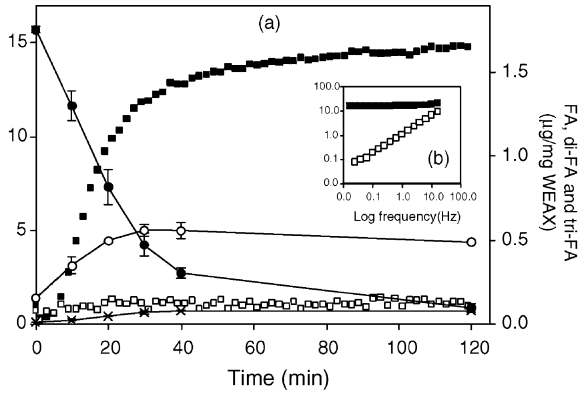

Fig. 1. (a) Monitoring G' (■), G'' (□) and ferulic acids (FA (●), di-FA (○), tri-FA (×)) as a function of time during 1% (w/v) WEAX solution gelation by laccase collected at 1 Hz, and (b) mechanical spectrum of the freshly cured gel. Data obtained at 25 °C and 10% strain.

During WEAX gelation, all di-FA did not vary in the same way and extent. At the end of gelation, the 8-5′(principally benzofuran form) 8-*O*-4′ and 5-5′ dimers represented 80, 16 and 5% of the total di-FA amounts, respectively. The main increase in di-FA concerned the 8-5′ benzofuran form. The predominance of 8-5′ and 8-*O*-4′ dimers and absence of the 8-8′ structure was also observed in wheat, barley, rye and triticale WEAX treated with peroxidase/H_2O_2 system (Dervilly-Pinel et al., 2001; Schooneveld-Bergmans et al., 1999) or laccase (Vansteenkiste et al., 2004).

The amounts of di-FA and tri-FA produced during gelation never counterbalanced the loss of FA. Indeed, at the end of gelation, 95% of the initial FA in the WEAX solution has disappeared, while only 24% was recovered as di-FA and tri-FA. Dervilly-Pinel et al., 2001; Schooneveld-Bergmans et al., 1999; Vansteenkiste et al., 2004 also reported low ferulate recovery after oxidative treatment of arabinoxylans. Similar results were obtained on feruloylated sugar beet pectin (Oosterveld, Grabber, Beldman, Ralph, & Voragen, 1997). These authors suggested the formation of higher oligomers of ferulate. In our study, trimers were actually created but other structures not detected here could explain the low FA oxidation products recovery.

It is well known that under oxidative conditions phenols can form relatively stable semiquinone or phenoxy radicals, which can be directly observed by EPR spectroscopy (Bors, Michel, & Stettmaier, 2000). The free radical generation in laccase-treated WEAX was followed by EPR during all gelation process (Fig. 2a) and for 24 h during gel aging

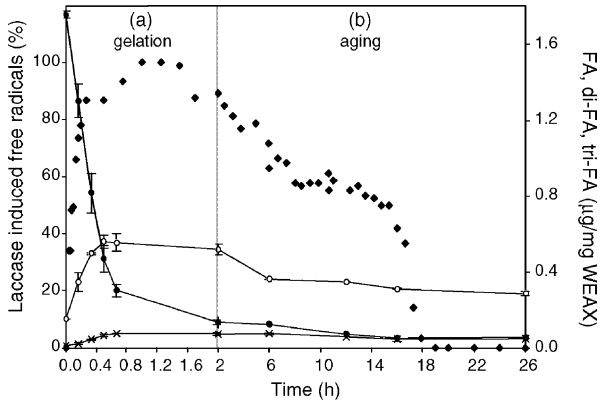

Fig. 2. Laccase induced free radicals (♦), FA (●), di-FA (○) and tri-FA (×) changes during: (a) 2 h of 1% (w/v) WEAX solution gelation, and (b) 24 h of gel aging at 25 °C.

(Fig. 2b). A radical signal (Fig. 3a) could be detected immediately after the 2 min period corresponding to the mixing of laccase with the 1% (w/v) WEAX solution and to the sample transfer into the EPR tube. This signal exhibited a fast increase in the first 30 min followed by a slower increase leading to a maximum amplitude after about 1 h incubation (Fig. 2a). The radical concentration corresponding to this maximum was determined by double integration of the signal: 0.60±0.06 μM. As the radical signal was stable for several hours, attempts to improve the spectral resolution by using a high number of accumulations and by decreasing the modulation amplitude were performed (Fig. 3b). However, no hyperfine structure was observed, even for modulation amplitude as low as 0.04 mT. This is likely due to unresolved hyperfine couplings related to the high number of protons in FA and to a signal broadening arising from the slow radical motion in the high viscosity WEAX gel.

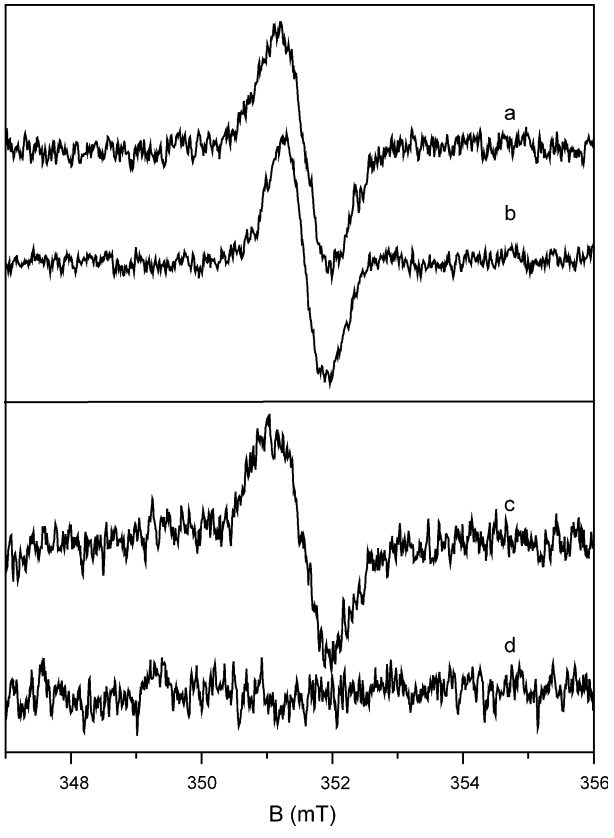

Fig. 3. EPR spectra of the laccase induced free radicals: (a) after 1 h of 1% (w/v) WEAX gel formation recorded with 10 accumulations (acquisition time of 6 min 40 s) and an amplitude modulation of 0.8 mT, (b) recorded with 1200 accumulations (acquisition time of 13 h 20 min) and an amplitude modulation of 0.1 mT. Effect of laccase thermal inactivation (90 °C–15 min) on the radical signal, (c) before thermal treatment, and (d) immediately after thermal treatment.

It is worth noting that the rapid radical production observed in the first 30 min and leading to 90% of the maximal signal corresponds to the maximal di-FA and tri-FA formation (Fig. 2a) and to the 80% of the final G' value (Fig. 1a). The following slower increase in the radical signal detected between 30 and 60 min can be related to the slight diminution in di-FA levels (from 0.56 to 0.49 μg di-FA/mg WEAX) and to the increase in G' (from 11.8 to 14.6 Pa) observed from 30 to 120 min (Fig. 1a). These results may suggest a radical mediated transformation of di-FA structures into higher molecular weight compounds.

3.3. Aging of WEAX gel

The rheological and biochemical stability of a freshly cured (2 h) WEAX gel were followed for 6 days at 25 °C. Gel rheological properties were greatly affected. Forty-three percent loss in the gel hardness was recorded between 2 h and 6 days. This rheological evolution could be partly due to the slight changes in the WEAX chain structure, i.e. the decrease in M_w and η_{red} of 20 and 17%, respectively (Table 2).

Arabinoxylan molecular weight was reported to influence the final WEAX gel strength (Izydorczyk & Biliaderis, 1995; Rattan et al., 1994). However, ferulic acid content and distribution on arabinoxylan chain and, as a result, the di-FA cross-links formation are also known to play a major role in

Table 2
Effect of 6 days storage at 25 °C on the rheological and physicochemical properties of WEAX gels

	Initial time (t=2 h)		At the end of storage (t=6 days)	
	Control gel	Heat treated gel	Control gel	Heat treated gel
Hardness, H (N)[a]	0.28±0.01a	0.22±0.01b	0.16±0.01c	0.21±0.01b
WEAX molecular weight, M_w (kDa)[b]	483±18a	490±41a	388±12b	468±56a
Reduced viscosity, η_{red} (ml/g)[b]	668±3.5a	610±0.8b	554±3.6c	606±0.7b

Uncommon alphabets along row indicate statistically significant differences ($P<0.05$).
[a] WEAX gels at 2% (w/v).
[b] WEAX gels at 1% (w/v).

Table 3
Changes in FA, di-FA and tri-FA (μg/mg WEAX) in control and heat treated 1% (w/v) WEAX gels for 6 days of storage at 25 °C

Time (h)	Control gel			Heat treated gel		
	FA	Di-FA	Tri-FA	FA	Di-FA	Tri-FA
2	0.134±0.002	0.494±0.020	0.074±0.001	0.120±0.005	0.530±0.021	0.078±0.001
26	0.055±0.002	0.287±0.010	0.049±0.001	0.126±0.006	0.466±0.022	0.082±0.001
50	0.041±0.002	0.240±0.012	0.040±0.002	0.133±0.002	0.468±0.007	0.080±0.003
98	0.026±0.001	0.191±0.008	0.030±0.001	0.132±0.007	0.478±0.018	0.085±0.004
146	0.019±0.001	0.161±0.004	0.020±0.001	0.125±0.001	0.470±0.001	0.069±0.001

All values are average from two repetitions.

the rheological properties of the WEAX gel (Figueroa-Espinoza et al., 1998a; Izydorczyck & Biliaderis, 1995). The changes in FA, di-FA and tri-FA in the WEAX gels along 6 days at 25 °C are showed in Table 3. FA, which mainly disappeared during the gel formation (95% decrease), was still oxidized until almost total disappearance from 0.134 (2 h) to 0.02 μg/mg WEAX (6 days). The FA decrease did not lead to the formation of identifiable cross-links. Indeed no di-FA nor tri-FA were created or even stabilised during aging. On the contrary, like FA, they rapidly decreased during the first 24 h of aging and then more slowly reaching 33 and 27% of the values at 2 h for di and tri-FA, respectively. In the 6 days WEAX gels, 8-5′, 8-*O*-4′ and 5-5′ structures were in a ratio of 84, 11 and 5% of total di-FA content, respectively. These values are close to those observed at 2 h showing that di-FA decrease concerned similarly all the dimers.

As shown in Fig. 2b, after gel formation (2 h), the radical signal decreased slowly during the next 16 h before vanishing suddenly under EPR detection limits (∼50 nM). According to Kurek et al. (1997), laccase oxidation of lignin phenolic compounds result in phenoxy radicals formation, which undergo non-enzymatic evolution leading to various C–C and ether bond cleavages in the polymer. By analogy, such radical-induced cleavage may occur in the WEAX gel. The radicals produced could be transfered through the WEAX network and cleavage at the cross-linking points and in the arabinoxylan backbone may occur. Indeed, in 1% (w/v) WEAX solution where ferulic acid was chemically eliminated by lime (unpublished data) no significant changes in η_{red} were observed after 6 days storage at 25 °C (0.3% η_{red} loss). Similar results were obtained when the deferuloylated WEAX solution was stored with laccase (2% η_{red} loss). On the contrary a 14% reduction in η_{red} was observed when the same deferuloylated WEAX were incubated in presence of laccase and free ferulic acid. These results confirm the role of oxidized ferulic acid as an initiator of the radical-mediated degradation of WEAX chain.

Laccase inactivated WEAX gels. In order to assess the role of laccase in the changes occurring during WEAX gel aging, a freshly cured WEAX gel was heat treated (90 °C–15 min) to inactivate the laccase. The thermal inactivation of laccase led to the total disappearance of the EPR signal in the gel (Fig. 3c and d). However, the heat treatment induced a loss in the gel hardness, which cannot be attributed to WEAX chain degradation since molecular weight distribution remained unchanged (Table 2). Physical rearrangements of WEAX chains more probably occurred, as reflected by a decrease in η_{red} values (Table 2). Heating a 1% (w/v) WEAX solution (same heat treatment and 1 h recovery at 25 °C) also led to a η_{red} decrease (670 to 472 ml/g) while molecular weight distribution did not change. Andrewartha, Phillips and Stone (1979) observing the same behavior in wheat WEAX solutions (boiled for 15 min) attributed this viscosity decrease to a conformational change in the WEAX chains. In our study, this effect was less pronounced in the WEAX gels state (η_{red} from 668 to 610 ml/g) than in solution because of the rigidity of the network.

As showed in Table 2, the thermal treatment allowed the mechanical properties of the gel to stabilize along the 6 days as measured by hardness value. Indeed, unheated gel exhibited a 43% loss of hardness, which was reduced to 5% loss when the gel was preliminary submitted to thermal treatment. The protective effect of heat against deterioration was also observed on WEAX chains characteristics (molecular weight and η_{red}) (Table 2).

In the same way, phenolics were stabilized with FA levels only varying from 0.120 (0 day) to 0.125 μg/mg WEAX (6 days), di-FA from 0.53 (0 days) to 0.47 μg/mg WEAX (6 days) and tri-FA from 0.078 (0 day) to 0.069 μg/mg WEAX (6 days) (Table 3). The relative percentages of 8-5′, 8-*O*-4′ and 5-5′ dimers were not modified by thermal treatment, representing 79, 16 and 5% of the total dimer content, respectively. These results indicate that FA, di-FA and tri-FA degradation during WEAX networks storage can be stopped by laccase inactivation in the freshly cured gel. Phenolic degradation reactions occurred because both FA compounds and O_2 were still accessible to the enzyme in the gel. As a fact, laccase (67 kDa) is able to diffuse through the WEAX gel network (unpublished data).

4. Conclusion

WEAX gel degradation observed after 6 days of storage appeared to proceed through a free radical mechanism initiated by laccase. The phenoxy radicals produced underwent secondary reactions resulting in a loss of WEAX cross-linking bonds and a partial depolymerization of the WEAX chains, which led to a decrease in gel hardness during storage. Laccase thermal inactivation just after gel formation, in spite of diminishing the network hardness initial values, stopped the free radical production and stabilized the cross-linking bonds and the gel hardness during aging. The WEAX gel degradation mechanism could involve two pathways:

(a) Laccase or laccase produced mobile radicals would attack the OH of the di-FA and tri-FA WEAX cross-linking structures. The uncoupled radicals formed could partially result in the formation of decarboxylated (mono, di and/or tri-) ferulate structures as reported to occur with free FA (Ward et al., 2001). This ferulate decarboxylation reaction implies the release of (mono, di and/or tri-) ferulate structures from the WEAX chain (cleavage of arabinose-FA linkage) resulting in a loss in the effective WEAX gel cross-linking bonds. The decarboxylated derivatives formed would therefore be able to undergo coupling with other laccase induced phenoxy radicals to form oligomeric structures as reported to occur during free FA (Ward et al., 2001) and lignin (Fournand, Cathala, & Lapierre, 2003) polymerization.
(b) Secondary free radicals issued from the initial FA oxidation by laccase, would attack the WEAX xylan chain resulting in a WEAX molecular size reduction. This WEAX xylan chain cleavage mechanism could be similar to that reported in polysaccharide degradation by hydroxyl radicals (Fry, 1998; Gilbert, King, & Thomas, 1984; Henriksson, Johansson, & Pettersson, 2000; Schweikert, Liszkay, & Schopfer, 2002). In our study, the addition of free FA in a deferuloylated WEAX/laccase system induced a significant loss in the η_{red} of WEAX confirming the possible role of phenoxy radical produced by FA oxidation in the WEAX chain degradation. The two degradation pathways (a and b) could co-exist in our laccase induced WEAX gel system.

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