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Title

Relationship between the structure of Fe-Lignosulfonate complexes determined by FTIR spectroscopy and their reduction by the leaf Fe reductase

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Introduction

Fe chlorosis is an old worldwide problem occurring in areas of calcareous and/or alkaline soils, and constitutes a limiting factor for agricultural production in many areas in the world. Nowadays synthetic Fe chelates are the most efficient remedy to control Fe deficiencies but natural Fe complexes, cheaper and more easily degradable, are under consideration. Lignosulfonates (LS) are pulp and paper industry by-products obtained during the sulfite pulping process, with good ability to form Fe complexes.

The aims of this work were to evaluate if structural changes occurred in the LS molecules when the percentage of complexing functional groups was modified and when Fe-LS complexes were formed, as well as its relationship with the reduction process that occurs in plant leaves. FTIR spectroscopy was used in the structural characterization of the LS molecule. Then, reduction of LS/Fe^{3+} by leaf reductase was tested.

Materials and Methods

Lignosulfonate sources

Hardwood and softwood LS samples produced by the sulfite process were obtained from LignoTech Ibérica, and their main characteristics are reported in Table 1. LS samples were obtained through sulfite treatment of hardwood (eucalyptus; LS1) and softwood (spruce; LS4) sources. LS2 and LS3 were obtained from LS1 by industrial modifications in order to increase the amount of functional groups of the polymer capable to complex Fe. LS/Fe^{2+} and LS/Fe^{3+} complexes were prepared by mixing LS with $FeSO_4$ or $FeCl_3$ (PA, PANREAC); the Fe:LS ratio was 1.0 µmol Fe and 1.1 µmol LS.

Table 1: Characteristics of the lignosulfonate sources (LS) tested

Sample	Wood type	Mw (g mol^{-1})	Org. S (g kg^{-1} LS)	-COOH (g kg^{-1} LS)	Phenolic -OH (g kg^{-1} LS)	Polysaccharides (g kg^{-1} LS)
LS1	Eucalyptus	6282	49	30	19	218
LS2	Eucalyptus	6954	49	60	19	22
LS3	Eucalyptus	7654	57	60	17	5
LS4	Spruce	32413	55	26	19	27

Fourier Transformed Infrared (FTIR) Spectroscopy

FTIR spectra were obtained on a FTIR Bruker IFS60v spectrophotometer with a MTC detector and diffuse reflectance (DRIFT) accessory. Spectra of LS samples were recorded in the region 4000-400 cm^{-1} at a resolution of 4 cm^{-1} in the transmittance mode, using the potassium pellet method.

Fe(III) reduction by leaf discs

Vacuum-infiltrated samples consisting of 40 leaf discs (5 mm diameter) isolated from young leaves of Fe deficient 25-days-old cucumber plants were incubated in 5 mL assay solution to measure Fe(III) reduction (LS/Fe^{3+} or EDTA/Fe^{3+} as substrate). In order to evaluate the influence of Fe nutritional status in reduction a control treatment with EDTA/Fe^{3+} and plants grown without Fe deficiency was established. Samples containing 40 leaf discs were washed twice for 10 min in 5 mL of 0.5 mM $CaSO_4$, 10 mM Mes-KOH (pH 6.0) and 1 mM KCl solution (Nikolic and Römheld, 1999; de la Guardia y Alcántara, 1996). Then the solution was replaced by the assay medium (0.5 mM $CaSO_4$, 1 mM KCl, 300 µM BPDS, 10 mM Mes-KOH (pH 6.0) and 100 µM LS/Fe^{3+} or EDTA/Fe^{3+}). After vacuum infiltration during 20

minutes (2 periods of 10 minutes) leaf discs were incubated for 60 min in continuous-orbital shaking in light at 25°C. Reduction rates were determined as formed $BPDS/Fe^{2+}$ by measuring the absorbance at 535 nm against blanks (without leaf discs) and using extinction coefficient of 22.14 mM^{-1} cm^{-1}.

Statistical analysis

Data were statistically evaluated using Analysis of Variance (ANOVA) with the program SPSS 15.0 and means were compared using Duncan's test at $P < 0.05$.

Results and Discussion

FTIR spectra of LS

The main bands observed are in agreement with those reported in other works for lignins, technical lignins and LS (Boeriu et al., 2004).
The FTIR spectra are shown in Figure 1. All the LS follow a common pattern, with a broad band 3600-3300 cm^{-1} corresponding to hydroxyl groups in phenolic and carboxylic acids and several bands with variable intensity in the fingerprint region (1900 to 800 cm^{-1}). In this region the main features appear at 1770 cm^{-1} (aromatic acetoxy groups), 1715 and 1630 cm^{-1} (unconjugated carbonyl-carboxyl stretching), 1600 to 1500 cm^{-1} (C=C skeletal vibrations), 1470-1460 cm^{-1} (C-H deformation combined with aromatic ring vibrations), 1260 cm^{-1} (C=O stretch), 1230-1215 cm^{-1} (C-C, C-O and C=O stretching), 1140 cm^{-1} (C-H in plane deformation), 1050 cm^{-1} (complex vibration associated with the C-O, C-C stretching and C-OH bending in polysaccharides) and 840 and 810 cm^{-1} (C-H out-of-plane deformations). The band appearing at 620-660 cm^{-1} is assigned to the sulfonic groups (S-O stretching vibration) formed from the reaction of sodium sulfite with the secondary OH of the aliphatic side chain of lignins.
The intensity of several bands varies after the formation of the Fe complexes, indicating that structural changes occur in the molecule due to the addition of the metal. The bands at 2940, 2830, 1715, 1470, 1500 and 1050 cm^{-1} were always less intense after the complexation of Fe (as Fe^{2+} or Fe^{3+}). The lower intensity of these bands, associated with CH, carbonyl and carboxyl stretching, CH deformation, aromatic ring vibrations, C=C aromatic skeletal vibrations and the vibrations of C-O, C-C and C-OH in polysaccharides, may be related to the formation of Fe phenolates and carboxylates. The vibration associated to the S-O stretching is also lower after the addition of Fe, especially for Fe^{3+}. Some bands exhibit a higher intensity after complexation, though in this case this behavior is very dependent on the source of Fe added to the LS. An important increment is observed at 1630 cm^{-1} when Fe^{3+} is added that would be ascribed to a major intensity of the unconjugated carbonyl-carboxyl stretching, maybe due to the inclusion of the metal in these structures. This new feature might indicate a higher affinity of these groups for Fe^{3+} instead of Fe^{2+}. In the case of Fe^{2+}, relevant increases in intensity appeared at 1150-1140 and 810 cm^{-1}, associated to CH deformations (in plane and out of the plane, respectively) that would be favored in the presence of divalent Fe.
In general, hardwood (LS1, LS2 and LS3) and softwood (LS4) LS present a similar pattern, irrespectively of their different origins and characteristics. With regard to the modifications on LS1 to form LS2 and LS3 are reflected in the spectra at specific bands (2940, 1630 and 1230-1215 cm^{-1}) and confirms the major presence the enrichment of groups capable to complex Fe.
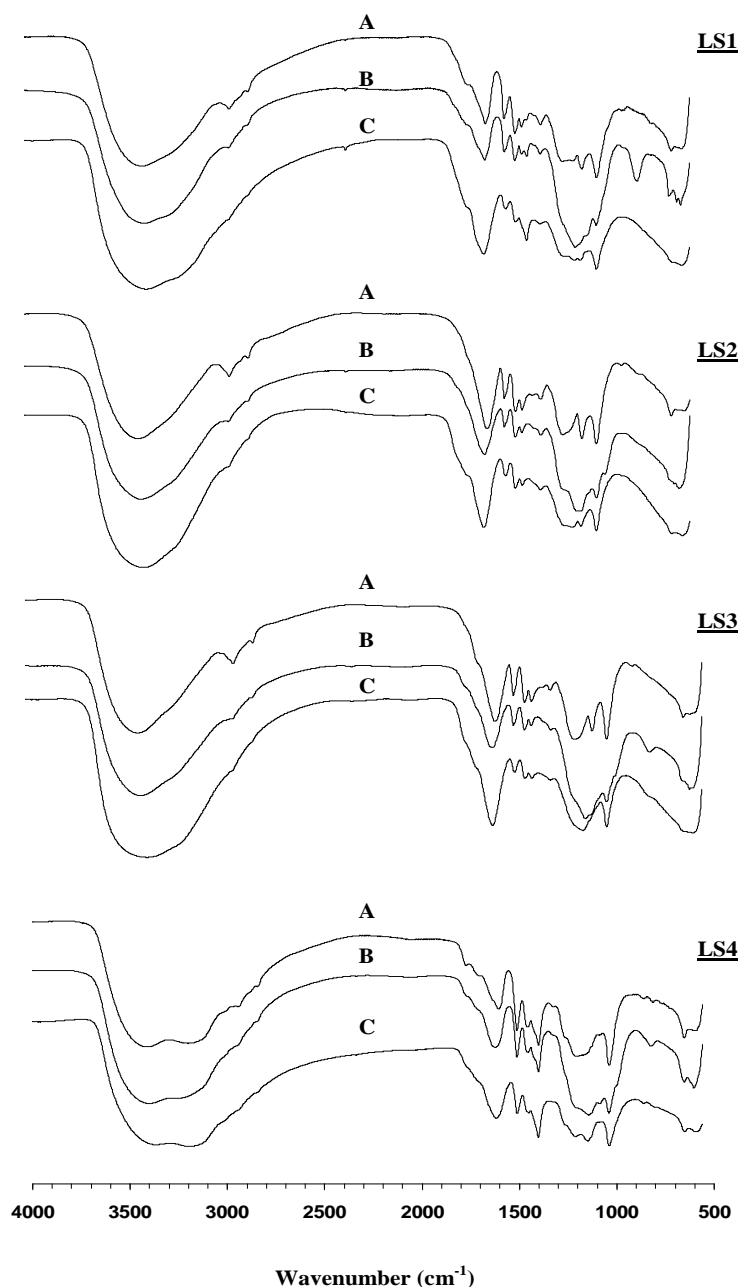
Figure 1: FTIR spectra of the lignosulfonates (A - LS, B - LS/Fe^{2+} complex, C - LS/Fe^{3+} complex).

Fe(III) reduction by leaf discs

The ability of leaf discs of cucumber Fe deficient plants to reduce LS/Fe^{3+} complexes in light was studied (Figure 2). The highest reduction rates corresponded to LS3/Fe^{3+}. The main features that seem to influence reduction rates were observed at 1630, 1140 and 1050 cm^{-1}. The band at 1630 cm^{-1} (carbonyl-carboxyl stretching vibrations) is more intense for LS3/Fe^{3+} than for the Fe^{2+} complexes and for the LS. At 1140 cm^{-1} (corresponding to CH in plane deformations) and 1050 cm^{-1} (related to complex vibrations associated with the C-O, C-C stretching and C-OH bending in polysaccharides) LS3/Fe^{3+}, LS4/Fe^{3+} and LS1/Fe^{3+} present higher intensity than LS2/Fe^{3+}. Thus, reduction was promoted in the complexes that CH in plane deformations (1140 cm^{-1}) and vibrations in the polysaccharides chain (1050 cm^{-1}). FTIR spectra indicated that after reduction stretching vibrations were less intense, while CH deformations in the plane were favored.
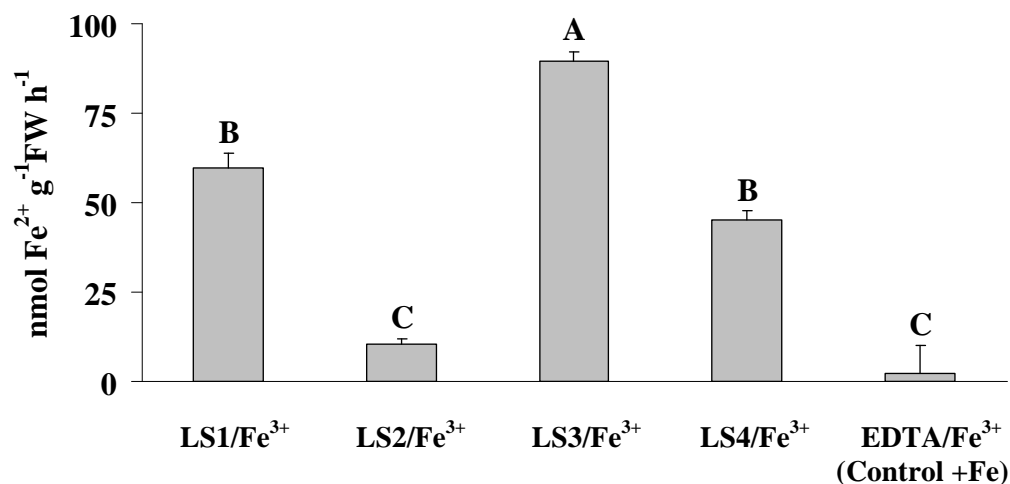
Figure 2: Reduction of LS/Fe^{3+} or $EDTA/Fe^{3+}$ by leaf discs of Fe-deficient cucumber plants. The measurements were performed in light. Data are means ± SE of five independent measurements.

Reduction of $EDTA/Fe^{3+}$ in light by Fe deficient plants was not observed, but when Fe sufficient plants were tested reduction of this synthetic chelate occurred although at levels lower than for LS complexes (except for the $LS2/Fe^{3+}$ complex). Photoreduction of $EDTA/Fe^{3+}$ might partly explain the low reduction associated to the reductase enzyme activity. On the other hand, this observation suggests that reduction is favored in non stressed plants.

In conclusion, FTIR spectroscopy can be an useful tool to elucidate the oxidation state of Fe and the molecular structure of Fe-LS complexes, as well as it can be used as an indicator of the effectiveness of LS complexes as leaf Fe reductase substrates. On the other hand, LS/Fe^{3+} complexes can supply Fe to leaf cells and constitute a promising Fe chlorosis corrector, but further research is necessary.

Acknowledgements

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